# ESTRADIOL INFLUENCES CENTRAL CONTROL OF BEHAVIORAL RESPONSES UNDERLYING BODY FLUID REGULATION

By

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Abstract: Extracellular fluid depletion and body sodium loss produces physiological and behavioral responses to restore body fluid homeostasis, and these responses are influenced by sex hormones. Sodium depletion produced by the natriuretic-diuretic, furosemide (FURO) stimulates salt intake that increases progressively with multiple FURO treatments, a form of behavioral plasticity that is more pronounced in females. Hormonal, osmotic, and neural signals regarding perturbations in sodium balance are initially processed within neurons and glia of the hindbrain dorsal vagal complex (DVC). Thus, plastic changes to glia and synaptic proteins can alter the effectiveness of synapses in the DVC to propagate signals to forebrain areas involved in body fluid regulation and thereby produce behavioral plasticity such as, progressive increases in salt intake. Our objective was to determine estradiol's effects on brain plasticity associated with the detection of peripheral changes during sodium loss. Accordingly, ovariectomized female rats with and without hormone replacement were used to test estradiol's effects on the 1) the time course of changes in glia in the hindbrain DVC, 2) the behavioral and physiological responses to repeated FURO-induced sodium depletion, and 3) the changes in expression of specific synaptic proteins in the hindbrain DVC after multiple FURO-induced sodium depletions. Adult female rats were ovariectomized, allowed to recover for 7 days, and then given estradiol benzoate (EB; 10 μg/0.1 ml oil, sc) or oil vehicle (OIL; 0.1 ml, sc) each week for 3 weeks. Rats were given two s.c injections 1-hour apart of FURO (5 mg/kg, s.c.) or 0.15 M NaCl (ISO; 1.0 mL/kg). Separate groups of rats were used for immunohistochemical analysis of glial fibrillary acidic protein (GFAP) in the dorsal vagal complex 2 h and 18-24 h after FURO/ISO, for urinary sodium and volume excretion and water and salt intake after weekly FURO/ISO treatments, and for western blot analysis of GFAP, β-actin, and the synaptic protein calcium calmodulin kinase II (CaMKII). Water and salt intake was increased in furosemide treated rats compared to isosaline treated rats independent of number of depletions. Estradiol produced a greater increase in salt intake compared to oil-treated rats after first furosemide-induced sodium depletion. Repeated furosemide treatment altered the expression of GFAP and CaMKII in the DVC. Estradiol may alter expression of GFAP and CaMKII in the DVC.

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# CHAPTER I

## INTRODUCTION

Females undergo profound physiological changes throughout the reproductive cycle and in pregnancy that differentiate them from their male counterparts and are evolutionarily necessary to the continuation of their species. It should be no surprise then that differences exist in complex regulatory processes governing the physiology between the sexes. Yet, research into sex differences has been slow to come about and far too many foundational studies concerning health and disease have been based on male-only populations. For instance, examination of hospital admissions of patients treated for cardiovascular disease with biologics that alter body fluid dynamics, such as diuretics, has revealed that adverse reactions to drugs differs between the sexes and this difference may be due to increased pharmacological effects in females (Rodenburg 2012, Juul 2011). The complex physiological mechanisms underlying these differences is yet to be determined. Therefore, it is vital that scientific research consider sex as a variable in studies outside of reproductive physiology, like body fluid regulation, as it may impact the cardiovascular and renal health and the treatment of these diseases in females.

Body fluid regulation is the maintenance of body water and body sodium involving independent and interdependent central and peripheral systems. For this reason, salt impacts life more than any other mineral. Despite the debate over the harmful effects of excess salt, too little salt or sodium deficiency has detrimental effects on body fluid balance, neural communication, and even successful reproduction (Moinier 2008, Morris 2008, Lesham 2009).

Body sodium is regulated to ensure the appropriate volume and content of both water and sodium are maintained for survival. For example, sodium loss induced by treatment with furosemide (FURO), a natriuretic-diuretic, increases salt intake and circulating angiotensin levels (Jalowiec 1974, Fluharty 1983, Fregly 1983, 1985, Lundy 2003, Moe 1984, Rowland 1999, Sakai 1986, Lind 1984). The compensatory behavioral response of ingesting sodium is controlled by the central nervous system in order to regulate blood sodium concentrations, fluid volume, and blood pressure. The central nervous system receives neural and hormonal input regarding perturbations in sodium balance from the periphery, synthesizes those inputs, and responds by altering motor, hormonal, and neural output to maintain homeostasis. Areas of the brain involved in this circuit include circumventricular organs, specialized areas that have an incomplete blood-brain barrier due to the presence of fenestrated capillaries that, together with astrocyte foot processes, control movement of ions and hormones into the brain parenchyma. Sensory circumventricular organs capable of detecting changes in plasma osmolarity and responding to peripheral hormones like angiotensin II during fluid challenges are in the forebrain, the organum vasculosum of the lamina terminals (OVLT) and the subfornical organ (SFO) (Antunes 2004, Daniels 2009), and in the hindbrain, dorsal vagal complex, which includes the nucleus of the solitary tract (NTS) and area postrema (AP). Together they serve as the sensors and integrative sites for stimulating compensatory behavior by relaying peripheral signals to areas of the hypothalamus, such as the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). The PVN and SON complete the circuit by producing hormonal signals that are then secreted either centrally or peripherally to initiate physiological and behavioral responses to fluctuations in body fluid balance. However, it has been difficult to identify central areas that specifically mediate behavioral responses such as salt intake to sodium loss. Thus, many investigators focus on areas involved in the detection of signal associated with salt loss such as that produced by FURO.

A particularly interesting aspect of FURO-induced salt intake is that repeated FURO treatments elicit progressively greater increases in salt intake (Sakai 1989), which occurs without

greater salt loss. Thus, the change in behavior with each experience of sodium loss suggests brain plasticity may be involved, an idea supported by the observation that dendritic spines in the nucleus accumbens increase in response to repeated sodium depletions. Moreover, rats having experience with sodium depletions display increased exploratory rearing compared to rats without a history of sodium depletions (Roitman 2002). The change in behavior with repeated episodes of sodium loss suggests brain plasticity may be involved in the detection and integration of signals stimulating salt intake.

Though plasticity is often associated with recovery of function after debilitating injury to the central nervous system, long-term alterations to central nervous system function and morphology also may alter physiological and behavioral responses to changing life experiences. Importantly, accumulating evidence suggests that plastic changes in the central nervous system can be induced by sex hormones, and have functional significance. For example, structural changes in dendritic spines of adult hippocampal neurons in response to estradiol are thought to underlie changes in synaptic efficacy, thereby providing a mechanism for adaptive behaviors associated with learning and memory (Gould 1990, Woolley and McEwan 1993). Increasing salt intake with repeated sodium depletions suggests salt intake is an adaptive behavior to preserve sodium balance. Although the studies of the central control of sodium regulation have been conducted primarily in males, important sex differences in behaviors associated with sodium balance have been observed. Female rats drink less water than do males (Richter 1929), a behavioral change proposed to be due to hormones (Santollo 2015), and the associated fluid retention and physiological changes may be necessary to support successful gestation. Even when fluid balance is relatively stable, female rats consume more sodium than do male rats and under conditions of sodium need, consume sodium in excess of the amount required to restore homeostasis (Wolf 1982). Our laboratory previously reported estrogen-mediated effects on water intake during intracellular and extracellular dehydration (Jones 2009). Interestingly, a pronounced sex difference in salt preferences also occurs, suggesting that control of sodium ingestion

may be influenced by the organizational and activational effects of estrogen (Curtis 2004), with the latter potentially underlying long-term plastic changes in the central control of sodium balance.

## Statement of the Problem

Little is known about the central mechanisms underlying female behavioral responses in body sodium regulation. Therefore, could changes in synaptic efficacy via estradiol modification of neuronal or non-neuronal cells in the central circuit regulating body sodium regulation underlie the behavioral responses to sodium depletion?

## Purpose of the Study

The purpose of this study was to examine estradiol's influences on the central control of behavioral responses underlying body sodium regulation via plastic changes in areas associated with the detection of peripheral changes during sodium loss. The present series of experiments will use ovariectomized female rats with and without hormone replacement to examine the time course of changes in astrocytes in the hindbrain dorsal vagal complex after a single furosemide-induced sodium depletion, assess the behavioral and physiological effects after multiple furosemide-induced sodium depletions, and observe changes in the expression of specific synaptic proteins in the hindbrain dorsal vagal complex after a single and multiple furosemide-induced sodium depletions. Identifying the mechanisms by which estradiol produces physiological and behaviorally significant effects in body sodium regulation will benefit our understanding of sex differences in disorders of body fluid regulation.

# CHAPTER II

## REVIEW OF THE LITERATURE

A study into estradiol's influence on the central control of behavioral responses underlying body fluid regulation covers subjects encompassing broad and deep literature bases. The following review includes background information on body fluid regulation, including the physiological and behavioral responses to water and sodium loss as well as the neural circuits involved. Progress in understanding estrogen's influence on body fluid regulation is also incorporated and brain plasticity is introduced as a possible means for functional changes that alter behavior.

### Body Fluid Regulation

#### Overview

Body fluid regulation is the mechanism by which living systems apply neuroendocrine, autonomic, renal, and behavioral processes to balance the intake of water and electrolytes with the output, so that the intake always equals the output, thus maintaining the stability of the internal environment for proper functioning of cells and tissues (Saladin 2003). Stability is achieved by maintaining the volume and solute concentrations within that fluid, or osmolarity (Saladin 2003). Osmolarity is primarily determined by the concentration of sodium, thus water and sodium regulation are closely interconnected. Deviations in osmolarity and volume are

detected by central and peripheral sensors (Antunes-Rodriques 2014). The sensors send information about water and/or sodium imbalances to the central nervous system (CNS), which in turn integrates the stimuli, and activates behavioral and physiological mechanisms to conserve and restore water and sodium levels. Once balance is restored, feedback signals inhibit further activation, reset the system, and prevent hazardous lack or excess of water or sodium. While this homeostasis is vital for survival, changes in the environment that lead to repetitive experiences with water or sodium losses can disrupt homeostasis, threaten that survival, and prompt behavior that would normally be inhibited, such as the excessive intake of salt. Prolonged salt intake behavior has negative effects on fluid regulation that can be maladaptive and even lead to the development of pathological diseases, such as hypertension. Therefore, it is important to understand the central control of the behavioral responses to the physiological loss of water and sodium.

**Physiological Responses to Water and Sodium Loss**

Water and sodium loss were recognized as important determinants in health and disease by physicians early in the 19$^{th}$ century who made great strides in understanding fluid's functional role within the body in order to discover a means for treating patients suffering from circulatory collapse due to severe dehydration or blood loss. Pioneers in the field laid the theoretical foundation of cellular (Gilman 1937) and extracellular fluid compartmentalization and the dynamic involvement of neural, vascular, hormonal, and osmotic systems in body fluid regulation (for review see Fitzsimons 1979). Scientific advancements led to McCance's observations that when there is sufficient loss of fluid volume, hypovolemia, which shifts water and electrolytes out of the extracellular compartment, sodium is also depleted, hyponatremia, and the correction of

this imbalance involves both water and salt intake (McCance 1936). While Guyton *et al.* (1975) described that the physiological responses to hypovolemia and hyponatremia were controlled by an interplay between cardiovascular, renal, and neural systems, signifying the difficulty in separating the effects of hyponatremia from hypovolemia and isolating the stimulatory mechanisms.

In hypovolemia, cardiovascular, hormonal, and behavioral compensatory mechanisms are activated to maintain vascular volume and blood pressure (Fig. 1). A drop in volume and pressure is detected by stretch receptors in blood vessels and baroreceptors in the aorta and carotid sinus of the heart. Stimulation by these receptors and the sympathetic nervous system travels to the hindbrain of the central nervous system and are further propagated to the hypothalamus. The hypothalamus initiates the release of vasopressin from the posterior pituitary. Vasopressin is a peptide hormone that upon binding to its receptor, in the kidney increases water reabsorption, and in the vascular smooth muscle stimulates vasoconstriction in order to preserve circulatory volume (Saladin 2010).

In tandem with a loss of volume, the osmolarity initially increases stimulating receptors in the hypothalamus and kidney sensitive to osmolarity, or osmoreceptors (Verney 1947). Activation of osmoreceptors stimulates vasopressin release to also increase water reabsorption. Oxytocin, a hormone similar to vasopressin, is released from the posterior pituitary and increases sodium excretion. Together, decreased water loss and increased sodium excretion result in maintaining both plasma osmolarity and plasma volume. However, if the circulatory volume is restored without replenishment of sodium, the net effect is a dilution of osmolarity which stimulates compensatory mechanisms to conserve sodium along with water. Equally important to the maintenance of circulation is the renin angiotensin aldosterone system, a local hormonal system regulating blood pressure and body fluid balance.

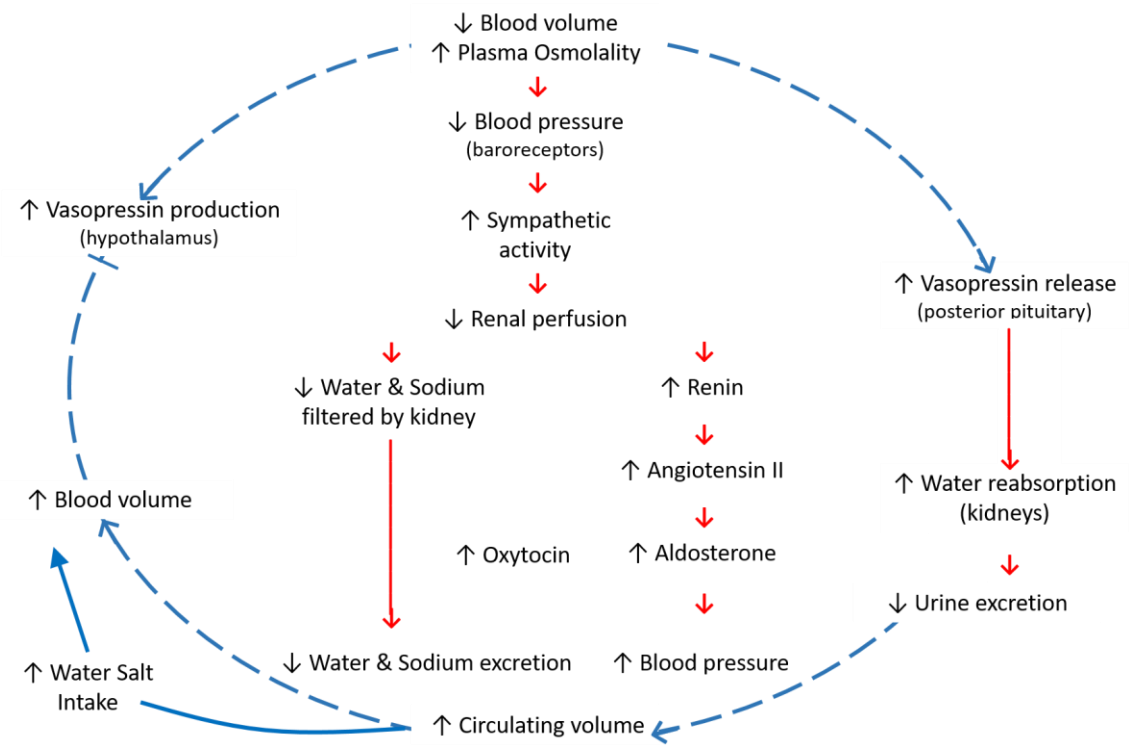


Figure 1. **Fluid balance via regulation of volume and osmolality.**

*The Renin Angiotensin Aldosterone System*

The renin angiotensin aldosterone system (RAAS) is also involved in the homeostatic control of blood pressure, tissue perfusion, and extracellular volume. In response to decreased blood pressure, volume, or osmolarity, the RAAS is activated. The RAAS signaling cascade begins with the production of renin made in the kidneys (Lavoie 2003). Exocytosis of renin occurs upon activation by a change in renal perfusion pressure detected by the arteriole baroreceptors and stretch sensitive juxtaglomerular cells in the kidney, a change in chloride concentration and delivery of sodium chloride to macula densa cells of the distal tubule in the kidney, or an activation of β-1 adrenergic receptors in the heart stimulated by the sympathetic nervous system [3]. Renin cleaves angiotensinogen secreted by the liver to form angiotensin I. Angiotensin converting enzyme (ACE) in the lungs hydrolyzes angiotensin I (Atlas 2007, Hsueh

1991) to form angiotensin II (Ang II). Angiotensin II is the active effector of RAAS and binds to angiotensin type 1 receptors (AT-1R). Angiotensin type I receptors are G-protein coupled receptors with subtypes found in the many cells of organs (AT-1a) and in the central nervous system (CNS) (AT-1b) associated with RAAS physiological and behavioral responses (Zhuo 2013). Binding of angiotensin II to AT-1 receptors leads to activation of multiple signal transduction pathways. The effects of these pathways varies but in general leads to vasoconstriction in the vasculature, increased blood pressure, and increased cardiac contractility.

In addition, angiotensin II and elevated plasma potassium ($K^+$) increases aldosterone release from zona glomerulosa cells of the adrenal glands. Aldosterone is a mineralocorticoid hormone that stimulates the reabsorption of sodium and excretion of potassium by binding to mineralocorticoid receptors in the kidney. By the reabsorption of sodium and potassium, water is indirectly conserved, thereby influencing volume and blood pressure. Angiotensin II also acts in the CNS to stimulate vasopressin release from the posterior pituitary in the hypothalamus and water and salt intake, discussed further below. Lastly, restoration of blood pressure and angiotensin II feedback onto the RAAS pathway serves to shut down the system by inhibiting the release of renin (Hall 2010).

**Behavioral Responses to Water and Sodium Loss**

While water and sodium conservation in the kidney is effective at maintaining body fluid balance in the short term, the only way to ultimately restore fluid levels is by ingesting water and sodium. Experiments on cellular dehydration found water deprivation and administration of hyperosmolar solutions were sufficient to stimulate water intake. Using these methods to disrupt

osmolarity, researchers were able to determine that the water and sodium loss is detected by central osmoreceptors and receptors for sodium (Verney 1947, Andersson 1953). The sites for these osmoreceptors are in the circumventricular organs, particularly the organum vasculosum of the lamina terminalis (OVLT) (Oldfield *et al.* 1994), the subfornical organ (SFO) (Watanabe 2005), and the median preoptic nucleus (MnPO) of the hypothalamus. Removal of any areas containing osmoreceptors damages their connections to vasopressin and oxytocin releasing cells in the PVN and SON, in turn attenuating drinking behavior in response to hypertonic saline infusions (Blass 1968, Coburn and Stricker 1978, Miselis, 1981). Even though drinking water can adequately restore balance in cellular dehydration (Wolf 1950, 1958), the same is not true in extracellular dehydration, where water intake further dilutes the extracellular compartment. In this state, homeostasis can only be restored by ingestion of sodium to restore the osmotic balance. Hence, the behaviors of both water and salt intake are stimulated under the control of the CNS in response to water and sodium loss. Although the mechanisms underlying salt intake behavior have yet to be fully explained.

Salt intake as a behavior is an enigma. Salt is physiologically needed by the body to function adequately and in that sense, it is understandable that animals would be motivated to seek salt in times of need. When salt is readily available though, and there is no deficiency to drive salt intake, animals continue to do so even to their detriment. In studies where animals were depleted of sodium followed by testing with varying concentrations of saline, depleted animals accepted saline solutions at concentrations that would normally be avoided. After a recovery, the animals' preference for saline solutions increased (Bare 1949, Smith and Stricker 1969). The shift from need to liking salt is the basis for a line of research trying to unravel the intricate physiological, behavioral, and psychological mechanisms underlying salt intake (McCaughey 1998, Morris 2008, Hurley 2015). In these studies sodium balance is disrupted via hypovolemia and/or hyponatremia to stimulate salt intake.

Concomitant with the loss of volume is the loss of sodium, together these stimuli activate the RAAS to produce aldosterone and ANGII. Aldosterone and ANGII act in the CNS to stimulate drinking and are used in the study of ingestive behaviors. Interestingly, antagonism of either central mineralocorticoid receptors (MRs) or angiotensin II receptors alone does not inhibit salt intake, but the combination of blocking MRs and administration of captopril, an ACE inhibitor, fully attenuates salt intake (Moe *et al.* 1984, Sakai & Epstein 1986, Fitts and Thunhorst 1996), demonstrating salt intake involves the cooperation/interaction between the aldosterone and angiotensin pathways. Consequently, resulting in the finding of a temporal disassociation between the stimulus to drink and the onset of drinking behavior. animals maintained on a sodium deficient diet show elevated aldosterone and ANGII (Menachery 1991) but will not consume solutions of saline until several days later. The delay in intake is not because of an inability to taste the saline (Curtis 2001) but may be due to an inhibitory drive.

Another stimuli for thirst during extracellular dehydration is hypovolemic activation of baroreceptors terminating in the NTS (Kaufman 1984, Zimmerman 1981). The NTS sends projections to the ventrolateral medulla and the lateral parabrachial nucleus, which in turn synapse with the SON and PVN, stimulating vasopressin and oxytocin release (Blessing *et al*. 1982, Cunningham, Jr. and Sawchenko 1988, Saper 1995, Saper and Loewy 1980). When plasma volume is normal, the NTS acts to inhibit the activity of this circuit, although the mechanisms of this inhibition are not fully understood. Although, the inhibitory actions of serotonin neurons in the dorsal raphe nucleus projecting on the SFO is involved in satiety during sodium ingestion following sodium depletion (Franchini 2002, Mecawi 2015).

Thus, many investigators focus on areas involved in the detection of signal associated with salt loss such as that produced by furosemide. A particularly interesting aspect of furosemide-induced salt intake is that repeated furosemide treatments elicit progressively greater

increases in salt intake (Sakai 1989), which occurs without greater salt loss. Thus, the change in behavior with each experience of sodium loss suggests brain plasticity may be involved, an idea supported by the observation that dendritic spines in the nucleus accumbens increase in response to repeated sodium depletions. Moreover, rats having experience with sodium depletions display increased exploratory rearing compared to rats without a history of sodium depletions (Roitman 2002). The change in behavior with repeated episodes of sodium loss suggest brain plasticity may be involved in the detection and integration of signal stimulating salt intake.

**Methods of Producing Water and Sodium Loss**

The study of body fluid regulation has benefited from techniques which allowed researchers to rapidly produce water and sodium loss. While chronically depriving animals of water and/or sodium (Swanson, Timson, & Frazier 1935) leads to an eventual state of depletion, it also has the negative consequences of generating stress and metabolic consequences that make deducing responses specific to fluid challenges difficult. Development of new techniques allowed for the determination of the contribution of key molecules, stimuli, and the organ systems involved in the physiological and behavioral responses to extracellular dehydration.

Early studies disrupted the osmotic balance by producing diuresis with the administration of sucrose (Holmes & Cizek 1951) that dropped the extracellular volume, resulting in water intake. Interestingly, water intake by the animals remained elevated until they were given salt suggesting sodium was necessary for the redistribution of fluid between compartments. In order to determine whether the water intake was being stimulated by the loss of volume or by the loss of sodium and whether the salt intake was stimulated by sodium depletion or secondarily from the

water intake, researchers employed the technique of peritoneal dialysis (Cizek 1951), which draws fluid out of circulation and exchanges osmolytes, thus disrupting osmolarity leading to volume depletion. By sequentially removing sodium from the diet, the relative responses to volume depletion or sodium depletion could be investigated. In experiments where rats were dialyzed with glucose then given a two bottle test consisting of one bottle of water and one bottle of 3% saline, dialyzed a second time, and then re-tested, rats showed an increased preference for saline but not water (Falk 1965). Still, this form of dialysis did not allow for the distinction between effects due to hypovolemia and hyponatremia.

Dialysis with a hyperoncotic colloid, either peritoneal (Fitzsimons 1961) or subcutaneous (Stricker 1966), is a rapid method of isotonic fluid depletion by creating an accumulation of fluid at the site of injection that is of the same osmolarity as that in circulation. Rats treated in this manner and subsequently given a two-bottle test of water and 1.8 % saline drank more water than saline initially, but when re-tested days later, drank greater amounts of saline (Fitzsimons 1971) (Stricker & Jalowiec 1970). Therefore, hypovolemia stimulated immediate water intake, but that intake could not compensate for the depletion because water alone served to further dilute body fluids. Only when osmolarity was reestablished by the ingestion of saline was fluid balance restored. Thus, salt intake was shown to be an independent behavior from water intake.

Pharmacological agents that activate the RAAS or disturb body fluid balance are often used to study the physiological and behavioral regulation of body fluids. For instance, β–adrenergic substances that lower blood pressure, such as isoproterenol, are used to study the mechanisms by which the components of the RAAS contribute to water intake. Alternatively, diuretics increase urinary excretion leading to extracellular dehydration. However, it has been difficult to identify central areas that specifically mediate behavioral responses such as

salt intake to sodium loss. Thus, many investigators focus on areas involved in the detection of signal associated with salt loss such as that produced by furosemide. Furosemide increases urine flow and urinary sodium loss within an hour after treatment, but an 18-24 hour delay typically transpires before rats consume sodium solutions. A particularly interesting aspect of furosemide-induced salt intake is that repeated furosemide treatments elicit progressively greater increases in salt intake (Sakai 1989), which occurs without greater salt loss. Understanding the shift in behavior towards salt intake not governed by need, requires studying the changes to the central control of the behavioral responses to water and sodium loss.

**Neural Circuits Involved in Water and Sodium Loss**

Perturbations in water and sodium balance are regulated by the central nervous system, which receives neural and hormonal input from the periphery, synthesizes those inputs, and responds by altering motor, hormonal, and neural output to maintain homeostasis. A key component in these processes is a set of privileged sensory organs, circumventricular organs (CVOs), within the brain. These specialized CNS organs have an incomplete blood-brain barrier, due to the presence of fenestrated capillaries that, together with astrocyte foot processes, control movement of ions and hormones into the brain parenchyma. Their location adjacent to the ventricles containing cerebrospinal fluid positions them to allow circulating hormones and peptides to access the CNS and for hypothalamic hormones to enter circulation without disrupting the blood brain barrier.

The dorsal vagal complex (DVC) in the hindbrain includes two sensory circumventricular organs, the nucleus of the solitary tract (NTS) and area postrema (AP), that share reciprocal

connections and make the DVC an important site for regulatory modulation. The AP is an easily accessed, highly vascularized nucleus on top of the medulla in the fourth ventricle just above the NTS. Structurally, the AP is made of small neurons, unmyelinated nerve fibers, and glia that receive blood through terminal arteries while capillary beds surround the spaces. Administration of fluorescently tagged proteins of varying sizes revealed diffusion was dependent on the size of the molecule, demonstrating the existences of a gradient within the extracellular spaces with larger molecules restricted within the AP and smaller molecules allowed passage (Willis 2007). The first studies into the function of the AP found a wide variety of chemicals and toxins introduced into the AP would cause nausea in the subjects, which led to it being labeled as a "chemical sensor" (Kelly 1982, Ossenkopp 1983, Borison 1989, Leslie and Reynolds 1991). AP lesion studies found an attenuation in feeding and drinking behaviors (Contreras *et al.* 1984) and after recovery, preferences for sodium and palatable foods appeared, suggesting the AP was involved in the inhibition of salt intake (Miselis *et al.* 1984, Edwards and Ritter 1981, Contreras and Stetson, 1981, Curtis *et al*. 1999, Edwards *et al.* 1993, Hyde and Miselis 1984, Wang and Edwards 1997, Watson 1985). Increased salt intake was also observed following infusion of angiotensin II (Watson 1986). Since then, the features attributed to the AP has grown to include the scope of autonomic systems, from cardiovascular, feeding and metabolism to body fluid regulation (Leslie, 1986, Borison 1989, Goehler *et al*. 2006). Modulating the physiological regulation of so many pathways is made possible by the absence of a complete blood brain barrier and the presence of countless receptors for detecting circulating hormones, such as angiotensin II and vasopressin. In essence, the AP becomes active, represented by c-fos neural activation or electrical activation, upon intrusion of an above threshold level of many molecules, including sodium (Clemente *et al.* 1957, Borison *et al.* 1975, Andreoli *et al*. 1980, Adachi and Kobashi 1985). Even peripheral infusions of sodium increase c-fos in the AP, whereas sodium depletion initiates no response (Miller and Loewy 2014, Miller *et al.* 2013). Research into the mechanisms of detecting sodium status revealed serotonergic neurons in the AP become activated after

hypertonic saline infusions (Miller and Loewy 2014) and this activation may involve epithelial sodium channels (Miller and Loewy 2014). However, it has been difficult to identify central areas that specifically mediate behavioral responses such as salt intake to sodium loss. Thus, many investigators focus on areas involved in the detection of signal associated with salt loss.

The NTS mediates changes in autonomic nerve activity and sensory detection of homeostatic pathways (Miselis 1984). The sensory neurons of the NTS are subdivided according to the function of the input it receives, with the rostral level associated with taste processing coming from the facial, glossopharyngeal, and vagus nerves, while the caudal level incorporates cardio-respiratory and gastrointestinal input. Projections from the NTS go to many areas in the brain including the paraventricular nucleus (PVN) in the hypothalamus, the amygdala, and within the brainstem, the AP, the parabrachial nucleus, locus coeruleus, and dorsal raphe nucleus (Andresen 1994, King 2007). A population of neurons in the NTS relevant to the control of sodium balance are the 11-β-hydroxysteroid-dehydrogenase 2 (HSD2) neurons, which have mineralocorticoid receptors (MR) for aldosterone (Geerling & Loewy 2006). During sodium depletion, aldosterone increases to reduce sodium excretion in the kidney, but its mechanism of action in the brain is less understood. Activation of MRs in the NTS increases blood pressure (Gomez-Sanchez 1986, Gomez-Sanchez *et al*. 1990), injection of the MR agonist, deoxycorticosterone acetate (DOCA) increases salt intake (Rice and Richter 1943), and aldosterone injections into the NTS induce a rapid salt intake (Formenti *et al.* 2012, Koneru *et al.* 2013). However, a recent study by Wang *et al.* 2016 showed that when spironolactone, an MR antagonist, was injected it failed to abolish the salt intake whereas administration of a G-protein coupled estrogen receptor (GPER) antagonist inhibited salt intake. Collectively, the results demonstrate that the HSD2 neurons in the NTS are important in the behavioral response to sodium loss, but the pathway of action is not yet clear.

Sensory circumventricular organs (Fig. 1) in the forebrain capable of detecting changes in plasma osmolarity and responding to peripheral hormones like angiotensin II during fluid challenges are the organum vasculosum of the lamina terminals (OVLT) and the subfornical organ (SFO) (Antunes 2004, Daniels 2009). Together they serve as the initial sensors and integrative sites for stimulating compensatory behavior by relaying peripheral signals to areas of the hypothalamus, such as the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). The PVN and SON complete the circuit by producing hormonal signals that are then secreted either centrally or peripherally to initiate physiological and behavioral responses to fluctuations in body fluid balance. Direct stimulation of the SFO with ANGII increases drinking and intravenous administration of ANGII increases c-fos, a marker of neural activation (McKinley 1992). Too, vasopressin and oxytocin secretion increases with the stimulation of the SFO (Ferguson & Kasting 1986, 1987, 1988, Holmes *et al*. 1987, Simpson 1981). Together, this established the SFO as an important relay center for regulation of integrated autonomic function.

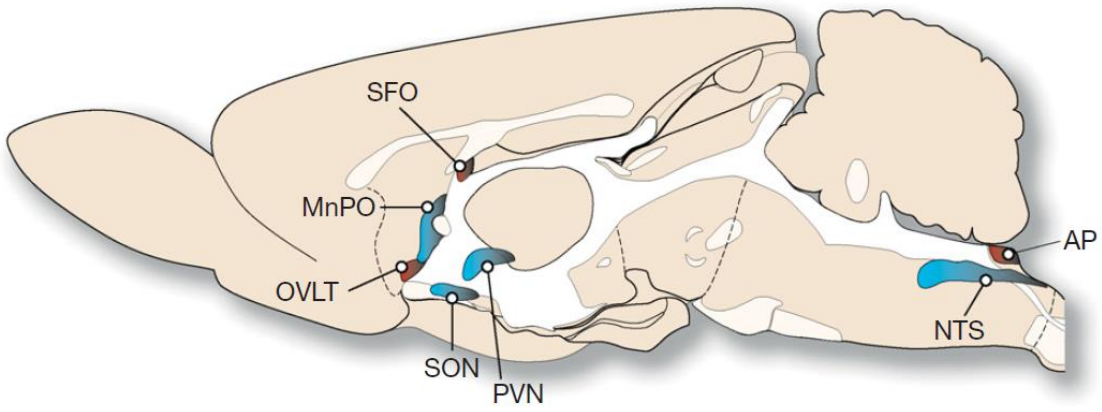


Figure 2. **Sensory circumventricular organs and AngII-sensitive sites in the brain.** Sensory CVO structures are shown in red and the location of several prominent AngII-sensitive sites are shown in blue. Reproduced with permission from Elsevier June 7, 2018; Daniels, D., and S. J. Fluharty. "Neuroendocrinology of Body Fluid Homeostasis-7." (2009).

## Brain Plasticity

Brain plasticity refers to physical changes in the structure of the brain that can alter its function. It includes changes to neurons or non-neuronal cells, like glia, that alter neurotransmission. Often brain plasticity is correlated with behavioral changes related to experience and can be affected by numerous factors such as, stress, age, and hormones.

### Synaptic Efficacy

Neural communication relies on the conversion of the electrical signal of an action potential to a chemical signal of neurotransmitter release or excitation-secretion coupling (Stahl 2013) across the synaptic cleft. Neurotransmitter is actively pumped into small endosomal packages called synaptic vesicles. Synaptic vesicles are stored in pools near active zones of the presynaptic terminal, where they are held in place by actin and synapsin I proteins until exocytosis (Sudhof 2004). Mitochondria provide the energy for synthesis of neurotransmitters, vesicle formation and trafficking. Sodium entering the axon terminal, or synaptic bouton, by opening of voltage sensitive sodium channels as the action potential propagates down the axon, activates voltage sensitive calcium ($Ca^{2+}$) channels (Stahl 2013). Calcium enters and binds to calmodulin. $Ca^{2+}$/calmodulin then binds to $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II) activating the kinase to phosphorylate synapsin I, in turn releasing the synaptic vesicle(Sudhof 2004, DeLorenzo 1981). Synaptic vesicles are coated in a large targeting protein complex of Rab GTPase proteins, soluble N-ethylmaleimide-sensitive factor attachment receptors (v-SNARE), and filament proteins which will traffic the vesicle to the plasma membrane using the cytoskeleton (Gundelfinger 2003, Haucke 2011, Bajjalieh 1999). The vesicular proteins,

synapsins, synaptobrevins, and synaptophysins also serve to recognize the presynaptic membrane, and aide in trafficking, fusion, and pore formation through interactions with presynaptic membrane proteins, syntaxin, physophilin, and t-SNARE (Sudhof 2004). Neurotransmitter exocytosis occurs when an increase in $Ca^{2+}$ at the membrane is detected by calcium-dependent activator protein for secretion (CAPS) and fusion is completed by synaptotagmin interacting with SNARE proteins (Bajjalieh 1999). Empty vesicles are either recycled or re-filled and re-used (Sudhof 2004), and once a neurotransmitter is released from the presynaptic terminal, it is degraded or removed from the synaptic cleft. Therefore, successful neurotransmission involves coordinated responses of many proteins and modulation of these proteins in turn can effect neurotransmission.

**Role of Glia in Synaptic Efficacy**

Though glia are best known for their contributions supporting the blood brain barrier, glia functions now include maintaining the synapse and promoting synaptic transmission. Early studies in frogs and mud puppies found a correlation between neuronal activity and changes in glia, when glial cells with no connection to neurons were depolarized after the associated optic nerve was stimulated (Orkand 1966). The depolarization was caused by $K^+$ released from neurons that had been taken up by the astrocytes (Walz 2000), pointing to a potential means by which glia can alter synaptic transmission by controlling ion concentrations in the extracellular space. Glial gap junctions, the intercellular connections between cells, have been found to facilitate the removal of ions, neurotransmitters, and metabolites from synapses (Barres 2008), and mediate the communication between the neuron and astrocyte (Froes 1999). Signaling between astrocytes in the form of calcium ($Ca^{2+}$) waves propagates via diffusion of inositol-1,4,5-triphosphate

($Ins(1,4,5)P_3$) (Sneyd 1995) and adenosine triphosphate (ATP) (James 2001) through gap junctions, and neuronal presence may up-regulate gap junction proteins in glia (Rouach 2000), providing a means for glia-neuronal communication.

The close association of neuroglia to neurotransmitter release sites (presynaptic membrane and postsynaptic membrane) form the tripartite synapse (Arauque 1999), and the presence of receptors for common neurotransmitters on neuroglia make them ideal to receive chemical information from neurons. Integration and modulation of neuronal activity can be seen when synaptic release of neurotransmitters such as glutamate (Porter 1996), acetylcholine, norepinephrine, and gamma-aminobutyric acid (GABA) (See review Hayden 2001) produces increased $Ca^{2+}$ in glia (Cornell 1990). Changing $Ca^{2+}$ levels is thought to 'activate' glial cells (Hansson 2003). Glial responses include propagating signals which may lead to long-term potentiation (Allen 2005), converting neurotransmitters like glutamate into glutamine or molecules that can then be re-used by neurons (Auld 2003), and activating release of astrocytic transmitters (gliotransmitters) to modulate synaptic transmission by activating receptors on neurons (Fiacco 2004, Liu 2004).

*Astrocytes*

Astroglia or astrocytes are a subset of glia that associate with every part of neurons, enveloping synapses and covering blood vessels, where they bring nutrients to neurons, aid in maintaining homeostasis by regulating ions and metabolites, serve as a barrier, and guide neural development (Hansson 2003, Hatten 1990). Astrocytes extend or retract their processes through the interactions with actin allowing them to change from a flattened shape to a stellate or star shape (Hatten 2002). Astrocytes express transporters and receptors enabling them to bind neurotransmitters or molecules, effecting diffusion and volume of the extracellular space (Hatten

2002, Sykova 2000). They can also have activational or modulatory roles in neurons through the release of gliotransmitters, including ATP and D-serine. ATP released from astrocyte lysosomes can be converted to adenosine, which then binds to presynaptic receptors on neurons to inhibit $Ca^{2+}$ channels, thereby reducing synaptic vesicle release of neurotransmitters (Zhang 2003). Astrocytic release of D-serine acts as an agonist for glycine binding sites on postsynaptic N-methyl-D-aspartate (NMDA) receptors (Mustafa 2004), thereby opening ion channels and activating calcium-dependent signaling cascades. Studies have also shown astrocytes release glutamate in neuron free cultures (Jeftinija 1996) and by inferring that glutamate detection near neuron-glia synapses (Bezzi 1998) or on oligodendrocyte precursor cells (Paukert 2006) after glia activation demonstrates astrocytic glutamate release. However transcriptome analysis has revealed astrocytes do not express any known vesicular glutamate transporters (Barres 2008). Therefore, further studies are needed to determine glutamate release by glia, the mechanism of release, and whether its function is to feedback onto neurons to regulate synaptic transmission. In conclusion, astrocytes and glia may be active participants in maintaining synaptic efficacy.

## Estrogen

### Overview

Estradiol is the predominant estrogen and is one of many fat-soluble steroid hormones derived from cholesterol that pass easily through cell membranes and participate in biochemical reactions (Nelson 2011) influencing the control of many physiological and behavioral processes. Estrogen's actions on reproductive physiology have been well studied and include the development of female secondary sexual characteristics, the initiation of changes in the female

corpus luteum, uteri, and genital tract (Nelson 2011), and the regulation of sodium transporters in the ducts of the epididymis of male testis effecting sperm concentration (Hess 2003). Reproductive physiology and mating behavior research led to the discovery of the sex hormones, testosterone (Brown-Sequard 1889) and estrogen (Diabetes 2005) and formulating the organizational-activational hypothesis to test differences observed between the sexes in physiology and behavior. Early studies such as these, broadened sex differences research to include studies in non-reproductive systems such as, the brain circuits (Remage-Healey 2014) involved in learning and memory (McEwen 1999), energy metabolism, neuroprotection (Arevalo 2015), and body fluid regulation (Curtis 2009). Studying estrogen influences on the central control of body fluid regulation will improve our understanding of how estrogen produces physiologically and behaviorally significant effects that may impact women's health.

**Estrogen Mechanisms of Action**

When studying estrogen effects on sex differences in physiology and behavior, consideration must be given to estrogen's organizational and activational effects. During embryological, fetal, and perhaps pre-pubertal development, estrogen's organizational effects act to permanently organize tissues so they differentially function upon gonadal hormone exposure later in life. For example, due to organizing effects of estrogen during development, estrogen is thought to protect female offspring from the effects of maternal hypertension during pregnancy and confer resistance to the development of early-onset hypertension in adulthood, whereas male offspring acquire no protection (Xue 2014, 2017). The activational effects are cyclical, acute, and reversible (Arnold 2009), as in the well observed effects during the female menstrual cycle when estrogen levels rise and fall over a relatively short time. Technological advances enabled scientists to study the cellular and molecular mechanisms that underlie the differences between

the actions of organizational and activational estrogen effects and demonstrate hormones act through many target tissues in separate and distinct processes (McCarthy 2008).

Estrogen effects produced over time with exposure involve estrogen acting on processes to change gene expression, whereas other effects are too rapid to be explained by genomic processes and utilize non-genomic methods to influence cell signaling. Estrogen exerts its genomic action by binding to its receptors (ERs), which are ligand-activated transcription factors. When estrogen binds to its receptor, the receptor trans-locates to the nucleus, changes its conformation, and complexes with coactivator proteins. The estrogen receptor complex then binds with an estrogen response element (ERE) in the promoter region of genes to modify transcription. Such direct modification is considered the classic method by which estrogen uses the nuclear receptors, ER-α or ER-β, to alter gene expression. Alternatively, estrogen can interact with other transcription factors, altering their expression or function to indirectly influence gene transcription. Regulating transcription is one of the ways estrogen acts to alter proteins, like receptors, and thus alter physiology. In reproduction, uterine contractions are stimulated when oxytocin binds to its receptor. Estrogen influences this process by increasing the expression of the oxytocin receptor (Heldring 2007).

Recently, rapid effects of estrogen have been recognized that cannot be explained by estrogen's genomic mechanisms. The findings that estrogen's non-genomic actions can modulate cell signaling (McEwen 2001, Gillies 2010) led to the discovery of the membrane estrogen receptor (mER) and the G-protein coupled receptor (GPER). Activation of membrane receptors revealed estrogen can alter intracellular second messengers, signal-transduction cascades, ion fluxes (calcium), cyclic AMP, and protein kinase pathways. Review of rapid estrogen signaling in the brain (see Raz 2008) Rapid effects of estrogen are seen when mice are tested for learning and brain plasticity and these effects are due to increase dendritic spine density and GPER enhancement (Gabor 2015).

**Estrogen Effects on the Control of Body Fluid Regulation**

Differences in the regulation of volume and osmolarity have been observed in pregnancy, across the reproductive cycle, and in hormone replacement therapy. Pregnant and lactating rats increase saline intake and maintain that increase throughout pregnancy (Richter 1938). Denton *et al.* observed increased salt intake in pregnant and lactating rabbits (Denton *et al.* 1971) and when estradiol or progesterone was administered to rabbits, salt intake only increased in rabbits given estradiol. Depriving lactating rats of sodium also produces increased sodium intake (Thiels 1990). Hence, the differences in salt intake were attributed to changes in the estrogen (Denton *et al.* 1971, 1973). Increases in thirst have also been observed in females prior to menstruation (Holmes 1967, Tarttelin & Gorski 1971) thought to be associated with premenstrual fluid retention and the variation in plasma volume across the estrous cycle (Slimmer 1996).

In general, female rats drink more saline (Krecek 1972, Richter 1929, Curtis 2004, Santollo 2015) than do males even when fluid balance is relatively stable. When body fluid balance is disrupted by osmotic challenges, estradiol has effects on water and salt intake (Curtis 2015). After hypertonic saline infusion, estradiol- treated rats respond by rapidly ingesting water (Jones 2009) and after sodium depletion, rats consume sodium in excess of the amount required to return to a balanced state (Wolf 1982, Sakai 1989). Such differences are due to the cyclic changes in the ovarian hormone, estrogen, and salt intake behavior shows sex differences, in part, due to differences in body weight (Santollo 2017). Estrogen is aromatized from androgen precursors by the enzyme aromatase in the brain (Roselli 1997) and reproductive organs of both males (Hess 1997) and females. Therefore, studying sex differences in target tissues and actions can help to explain the observed differences in physiology and behavior between males and females.

Behavioral changes in fluid intake by estrogen are mediated via interactions with estrogen receptors (ERα, ERβ, GPER) in key areas of the brain, including the hypothalamus (Santollo 2015). ER expression can be altered by osmotic stimuli, in turn impacting the effect of steroid hormones (Somponpun 2003). For example, the vasopressin (AVP) system shows sex differences via actions through ERs (Sladek 2008, Lucio-Oliveira 2015), as male rats have greater resting plasma AVP levels, greater AVP sensitivity and denser AVP projections (Carter 2009) and in females estradiol benzoate (EB) treatment lowers the osmotic threshold of AVP (Stachenfeld 2008). The consequences of these sex differences are females display increased salt intake and less AVP sensitivity to that intake while males display limited salt intake and are more sensitive to AVP.

Physiological differences in the control of circulation can also be seen in young women, who generally have lower blood pressure than young men (Joyner 2015) and after ovariectomy, when ovarian hormones are decreased and resting blood pressure increases. The changes are attributed to estradiol modulation of the autonomic nervous system, specifically in volume regulation by aldosterone and responses to ANG II (Toering 2017). Hence, sex differences in blood pressure and cardiovascular disease have been interpreted to arise from sex differences in the renin angiotensin aldosterone system (RAAS). Studies investigating estrogen's effects on the RAAS to mediate cardio-protective effects have produced conflicting results and continues to be masked in controversy. Estrogen's effects vary across the menstrual cycle, method of hormone replacement, or in the tissue being studied (Daniels 2009, Stachenfeld 2008). In general, estrogen has been found to increase the vasodilatory components of the RAAS, angiotensinogen, AT-2, Ang-(1-7), and ACE2, while decreasing the vasoconstrictive hypertensive components, renin, aldosterone, and ACE (Daniels 2009, Stachenfeld 2008, Komukai 2010).

In addition, estradiol acts on areas of the brain involved in the regulation of sympathetic outflow and blood pressure (Hay 2014). Baroreflex function is altered in females after estradiol

treatment, as demonstrated by the increases baroreflex sensitivity by modulation of ERα in the NTS (Spary 2010) and the correlated decrease in oxytocin in the PVN (DeMelo 2016), mediated by a decrease in ERβ expression (Somponpun 2004). Also in the hindbrain, neural activation decreases in areas involved in body fluid regulation by estradiol-treated rats (Jones 2012). Moreover, central infusions of ANG II induce water intake that may be regulated by estrogen treatment (Jonklaas 1984) mediated by increasing neural activation in PVN vasopressin neurons (Kisley 2000) and blocked with antiestrogen (Kisley 1999). Similarly, in reaction to ANG II or hypernatremia, estrogen changes the response of neurons in the SFO that project to the SON (Ciriello 2013). In regards to body sodium regulation, serotonin neurons increase after sodium depletion in rats treated with estradiol and has been linked to the inhibition of salt intake (Dalmasso 2011). Nonetheless, this cannot explain how estrogen influences the removal of this inhibition to allow salt intake, nor does it provide a mechanism for the enhanced salt intake observed in females. Investigations into estrogen modifications of salt intake behavior may benefit from studies comparing the expression of genes or proteins involved in the synaptic plasticity of neural circuits following water and sodium loss.

**Estrogen Influences on Synaptic Efficacy**

Synaptic efficacy centers on the strength of the synapse and relies on the functioning of each process of neurotransmission from the presynaptic terminal to the postsynaptic terminal. One way estrogen can affect synaptic efficacy is by altering the presynaptic release of neurotransmitter. An example of this can be seen when estrogen increases expression of vasopressin in areas of the brain influencing social memory (Fink 1996). Estrogen effects have also been discovered to impact synaptic efficacy by acting on interneurons where a decrease in the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Murphy 1998) alters

dendritic spine density (Woolley 1992) in hippocampal cells. Too, ER agonist activity shows estrogen regulates the expression of synaptic proteins (Waters 2009) and increases the number of synaptic boutons (Woolley 1996), thereby increasing synaptic efficacy. In the circuits of learning and memory, estrogen modulates synaptotagmins (Crispino 1999) and CAMKII (Sawai 2002). Could these proteins also be involved in estrogen mediated enhancement of salt intake behavior?

**Estrogen Influences on Glia**

Organizational estrogen exposure during radial glia formation, differentiation, and migration may impact the abundance of glia in distinct areas or preferentially guide differentiation to a particular type of glia in certain areas. Estrogen may act to promote the process of gliogenesis, or inhibit the processes of cell death, leading to increased numbers of glial cells. Developing glia may be uniquely influenced by estrogen, such that the phenotype of glia is altered or sensitized to change specific responses to growth hormones, neurotrophic factors, or second messengers upon adult exposure to estrogen through the regulation of receptor expression. For instance, I recall reading that there are sex differences in dendritic branching and the pruning process in adults. The mechanisms by which astrocytes are activated and participate in these processes may be modified during development by estrogen. Too, with the newly recognized implications of epigenetics, its possible effects of estrogen on transcription may be organizationally altered due to estrogen modulation of epigenetic mechanisms on chromatin to silence genes.

Activational responses to estrogen occur upon re-exposure and may include estrogen upregulation of estrogen receptors in glia to regulate glial functions, where estrogen binds to estrogen receptors on glia to produce changes in $Ca^{2+}$ signaling (Kuo 2010). Additionally, estrogen may also regulate the production of gliotransmitters either directly via estrogen receptor

mediated modulation of transcription or indirectly by regulating the production of the rate limiting enzymes for the transmitters. In this way estrogen can act through estrogen response elements at the promoter region of genes specific for glial receptors to induce activational regulation of glial neuronal communication. In response to brain injury, estrogen has been found to have protective effects, even participating in the repair process itself. Local production of estrogen by aromatase synthesis in astroglia is believed to aid in neural repair (Saldanha 2009). Studies investigating the effects of estradiol treatment on the development of neuro-psychiatric disorders found estradiol binding to ERα expressed on astrocytes had beneficial effects in experimental autoimmune encephalomyelitis (Spence 2011). Finally, the outcome of estrogen's effects may be a consequence of the summation of both organizational and activational effects. Still, little research has looked into the influence of estrodial on glia in response to water and sodium loss.

In summary, the brain is a powerful integrator of sensory information for regulating physiological processes with precision and urgency, including body fluid regulation. Yet, experiences and exposure to hormones change it in ways we still do not fully understand. Nor have we realized the impact brain plasticity may have on behaviors associated with regulated physiological processes. Investigating estradiol's influence on synaptic efficacy after sodium depletion will improve our understanding of the brain plasticity underlying the behavioral plasticity observed in enhanced salt intake.

# CHAPTER III

## MATERIALS AND METHODS

### Animals

White albino rats (*Rattus norvegicus*) of the Sprague-Dawley strain were purchased from Charles River or obtained from breeding colonies at Oklahoma State University Center for Health Sciences. Adult female rats weighing 220 – 375 g at the beginning of the experiment were individually housed in plastic cages in a temperature controlled (21-25°C) room with a 12-hr light-dark cycle and given *ad libitum* access to rodent chow (Harlan #2018) and water. All procedures were approved by the Oklahoma State University Center for Health Sciences Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Ovariectomy and Hormone Replacement

Rats were weighed and anesthetized with either sodium pentobarbital (Pbt; 50 mg/kg body weight (bw), i.p.) or isoflurane (2-2.5%; 1.5 -2 L/min flow rate). The ventral abdomen was shaved, disinfected with Betadine, wiped with alcohol, and treated with topical anesthetic (Bupivicaine; 5 mg/kg). A single longitudinal incision was made at the midline and the muscle incised to access the ovaries. Ovaries were bilaterally removed by first tying off each uterine horn, cauterizing the blood vessels, and then excising the ovary distal to the suture. The muscle was closed with sutures and the skin closed with stainless steel staples. Rats were given Meloxicam (1.5 mg/kg bw, p.o.) for postoperative pain management and allowed to recover. Seven days after

surgery, ovariectomized animals were given s.c. injections of the sesame oil vehicle (OIL; 0.1 ml) or 17-β-estradiol-3-benzoate (EB; Fisher Scientific; 10 μg/0.1 ml sesame oil) to mimic the pattern of estrogen fluctuations in reproductively intact rats (Curtis 2015). Specifically, rats were given EB or OIL injections between 09:00-11:00 on days 1 and 2 of a 4-day regimen (Figure 3).

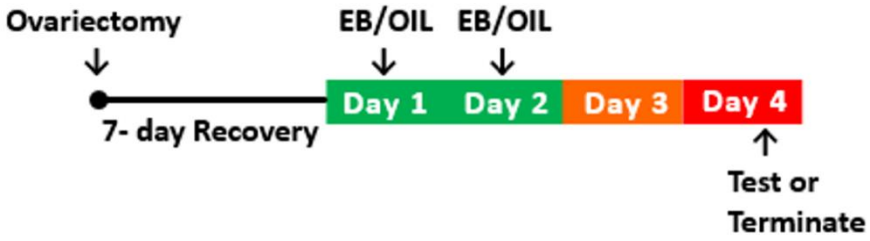


Figure 3. **Timeline of hormone replacement regimen.** Seven days after ovariectomy, rats received s.c. injections of EB or OIL between 09:00-11:00 on days 1 and 2 of a 4-day regimen. Rats were tested or terminated on day 4 as described in the experimental protocols.

Rats were weighed on each of these days and the efficacy of hormone replacement was determined by assessing the change in body weight (McCarthy 1991; Geary 1994; Graves 2011; Curtis 2015). As an additional verification of hormone efficacy, uterine weight was assessed at termination (Cooke 1997; Graves 2011; Miura 1971). Uteri were collected at sacrifice and stored in 0.15 M NaCl. Adipose was removed from the uteri, a 10-mm segment of uterus was cut near the bifurcation of the uterine horn, and then weighed.

**Furosemide Treatment**

Between 09:00-11:00 am on the morning of injections, food and water were removed from cages and OIL- and EB- treated rats were randomly assigned to be given two s.c injections of furosemide (FURO; 5 mg/kg bw) or 0.15 M NaCl (ISO; 1.0 mL/kg bw) separated by one hour (Sakai 1989). Rats were weighed before each injection and, following injection, returned to clean

cages. Testing or termination after FURO treatment followed three separate protocols. For one group of OIL- and EB-treated rats, rats were given injections of FURO or ISO on day four of the hormone regimen as described above. After the second injection, rats were returned to their cages for 2 h without access to food or water. Rats in this 2 h FURO protocol (Figure 4) were weighed and then either tested or terminated.

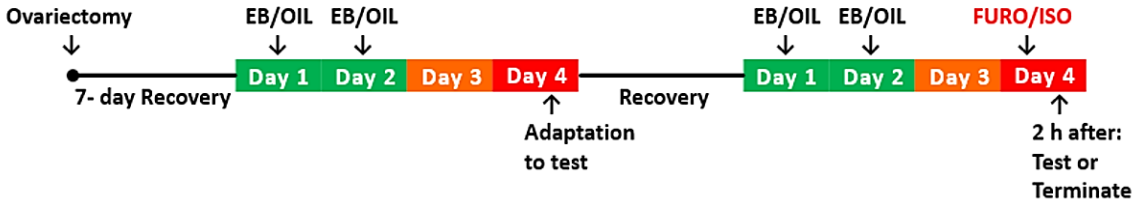


Figure 4. **Timeline of 2 h FURO treatment protocol.** Seven days after ovariectomy on day 4 of the hormone regimen, rats were adapted to the testing procedures. One week later, on day 4 of the hormone regimen, rats were given two s.c. injections of FURO (FURO; 5 mg/kg bw) or 0.15 M NaCl (ISO; 1.0 mL/kg bw) separated by one hour. Two hours after the second injections, rats were tested or terminated.

A second group of OIL- and EB-treated rats were given injections of FURO or ISO on day three of the hormone regimen as described (Figure 3). After the second injection, rats were returned to their cages for 18-24 h, during which they had access to water but not chow. On day four, rats in this single 18-24 h FURO protocol (Figure 5) were weighed and then either tested or terminated.

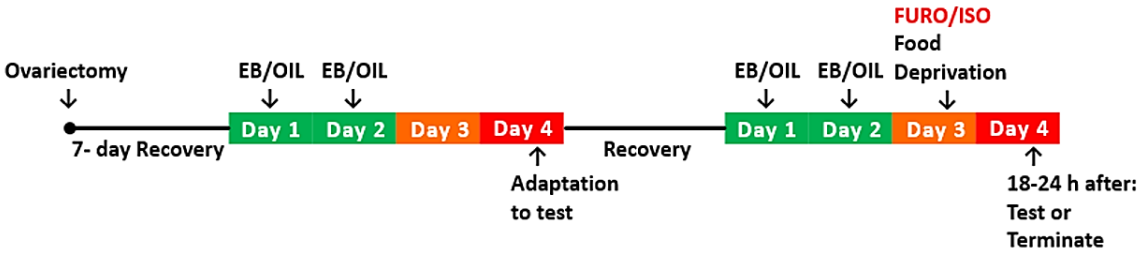


Figure 5. **Timeline of 18-24 h FURO treatment protocol.** Seven days after ovariectomy on day 4 of the hormone regimen, rats were adapted to the testing procedures. One week later, on day 3 of the hormone regimen, rats were given two s.c. injections of FURO (FURO; 5 mg/kg bw) or

0.15 M NaCl (ISO; 1.0 mL/kg bw) separated by one hour. 18-24 hours after the second injections, rats were tested or terminated.

A third group of OIL- and EB-treated rats were given FURO or ISO treatment on day three of the hormone regimen as described (Figure 4). On day four, rats in this multiple 18-24 h FURO protocol (Figure 6) were weighed and then tested. Following testing, rats were returned to cages and given *ad libitum* access to both food and water for three days. This procedure was repeated at weekly intervals for a second and third week. On day four of the third week, rats were tested or terminated.

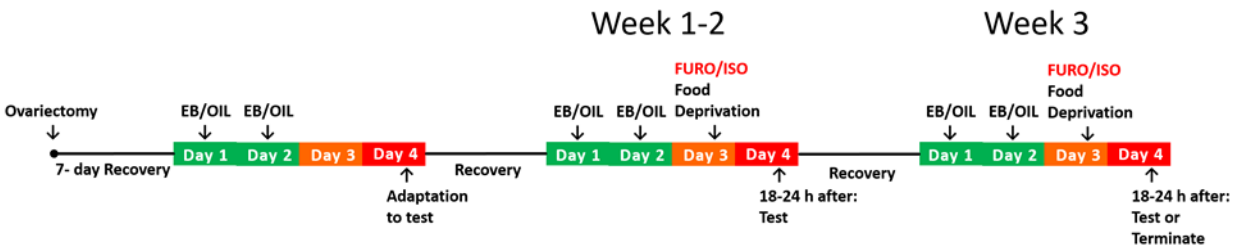


Figure 6. **Timeline of multiple 18-24 h FURO treatments protocol.** Seven days after ovariectomy on day 4 of the hormone regimen, rats were adapted to the testing procedures. One week later, on day 3 of the hormone regimen, rats were given two s.c. injections of FURO (FURO; 5 mg/kg bw) or 0.15 M NaCl (ISO; 1.0 mL/kg bw) separated by one hour. 18-24 hours after the second injections, rats were tested. This procedure was repeated at weekly intervals for a second and third week. On day four of the third week, rats were tested or terminated.

**Experiment 1: Furosemide Effects on Glia in the Dorsal Vagal Complex**

Experiment 1 compared changes in astrocytes in the hindbrain dorsal vagal complex of separate groups of OIL- and EB- treated rats given FURO or ISO treatment using the 2 h protocol (ISO: OIL n = 4-6; EB, n = 3-5; FURO: OIL n = 4-7; EB, n = 4-6) or the single 18-24 h protocol (ISO: OIL n = 2-3; EB, n = 3-5; FURO: OIL n = 4-5; EB, n = 3) as described (Figures 4 and 5). In this experiment, animals were added to increase the power groups of identically treated and processed

rats from previous FURO studies in our lab (Curtis 2015; Core 2017), using remaining tissue from a 1:3 series (see below for tissue collection).

*Perfusion, Termination, and Tissue Collection*

At sacrifice, rats were weighed and then anesthetized with sodium pentobarbital (Pbt; 50 mg/kg bw, i.p.). After rats failed to respond to tail and paw pinches, they were placed on a perfusion tray in a fume hood. The lower abdomen was incised and this incision was expanded through the diaphragm and ribs to expose the heart. Blood samples were collected from the heart into heparinized tubes for analysis of plasma protein, sodium, potassium, and osmolality; aliquots were collected into micro-hematocrit capillary tubes for analysis of hematocrit. Rats then were perfused transcardially with 0.15 M NaCl followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde overnight, and then transferred to a 30% sucrose solution, and stored at 4° C.

Uteri were collected and analyzed as described (Ovariectomy and hormone replacement). Blood samples were centrifuged at 5000 rpm for 20 minutes. Following centrifugation, plasma was aliquoted into Eppendorf tubes. One to two drops of plasma were analyzed for protein concentration using a refractometer (Leica). Remaining plasma was frozen at -80°C for later analysis of plasma sodium and potassium concentration using an ion-sensitive electrode system (EasyLyte). Plasma osmolality was determined using a Capillary tubes were centrifuged at 12000 rpm for 5 minutes. The ratio of the volume of red blood cells to the volume of the whole blood was calculated and used to determine hematocrit.

*Immunohistochemistry*

Fixed brains were cut in coronal sections at 40 μm in a 1:3 series through the forebrain and hindbrain using a cryostat (Leica) and then stored in cryoprotectant (Watson 1986) at -20° C until processed for immunolabeling. One of the three series of free-floating brain sections were

processed for glial fibrillary acidic protein (GFAP; a marker for astrocytes). Briefly, tissue sections were rinsed in 0.05 M Tris-NaCl, incubated in 0.5% $H_2O_2$ for 30 min, rinsed again, and then incubated in 10% normal goat serum (NGS) in 0.05 M Tris-NaCl with 0.5% Triton-X for 60 min. Tissue sections then were incubated overnight in primary antibody (Millipore: mouse anti-GFAP) diluted 1:6,000 in 2% NGS on a rocker at 4° C, then rinsed with 2% NGS and incubated in goat anti-mouse IgG (Jackson Immunoresearch; Cy3) diluted 1:200 in 2% NGS for 4-6 hours on a rocker at room temperature. After final rinses, sections were mounted on gelatinized slides, dried overnight, dehydrated in alcohol and xylenes, and then cover slipped using Cytoseal 60 (Fisher Scientific).

*Image Analysis and Quantification*

Viscerosensory information is initially integrated in the hindbrain dorsal vagal complex which comprises the nuclei of the area postrema, the nucleus of the solitary tracts, and the dorsal motor nucleus of the vagus. Because the dorsal vagal complex encompasses regions that can be subdivided based on function (Swanson 1980), immunohistochemical analysis was conducted within these subdivisions. The area postrema (AP; Bregma -13.68 to -14.08) and nucleus of the solitary tract (NTS; Bregma -13.68 to -14.60) were identified using landmarks describe by Paxinos and Watson (Paxinos and Watson 1998) under dark field microscopy using a Nikon 80*i* microscope fitted with a camera and rhodamine filter. Each area was traced under fluorescent illumination and, with exposure times held constant, mean intensity of GFAP immunoreactivity (GFAP-ir) within the outlined area was determined using NIS-Elements AR software 3.2 (Nikon). GFAP-ir mean intensity was assessed in rostral, middle, and caudal levels of the AP and in the caudal and middle levels of the NTS from each animal, with levels matched between animals. Group means for each area were calculated from the intensities.

**Experiment 2: Furosemide Effects on Measures of Body Fluid Balance and Behavioral Responses**

Experiment 2 assessed physiological and behavioral responses by OIL- and EB- treated rats (OIL, n = 4; EB, n = 5) given ISO and FURO treatment in a within subjects design using the multiple 18-24 h protocol as shown (Figure 7). Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1 and FURO treatment weekly for three weeks thereafter. After each treatment, rats were tested for urinary sodium and volume excretion and water and salt intake.

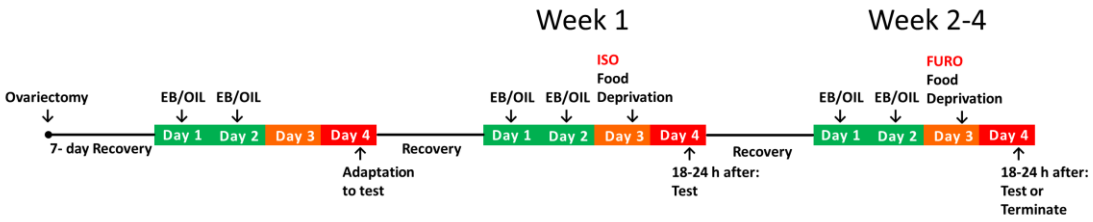


Figure 7. **Timeline of multiple 18-24 h FURO treatments protocol in a within subjects design.**

One week prior to testing, OIL- and EB- treated rats were weighed and placed in metabolic cages without food for 24 hours to adapt them to testing procedures and to collect baseline urine values. At the end of the 24 hours, rats were given 3-hr access to deionized water and 0.5 M NaCl (salt) in graduated drinking tubes to adapt them to the intake test. Following adaptation, rats were returned to home cages with *ad libitum* access to food and water. One week later, rats underwent ISO treatment on day 3 as described in the multiple 18-24 h FURO or ISO protocol (Figure 6). After each injection, rats were weighed and returned to metabolic cages without food or water. Urine was collected one hour after the first injection and again one hour after the second

injection. Rats remained in metabolic cages 18-24 h (i.e. until day 4) after the second injection with *ad libitum* access to water but no food. Urine was collected the following morning and water intake and body weight were recorded.

Between 09:00 – 12:00, rats underwent a two-hour two-bottle intake test. Intakes of salt and water were recorded after two hours, then rats were returned to home cages with *ad libitum* access to food and water. This procedure was repeated at weekly intervals for three additional weeks but with FURO injections on these subsequent weeks. Urinary sodium and potassium concentration was assessed using an ion-sensitive electrode system (EasyLyte) as described.

**Experiment 3: Furosemide Effects on Synaptic Proteins**

Experiment 3 investigated calcium calmodulin kinase II protein expression in the hindbrain dorsal vagal complex in separate groups of OIL- and EB- treated rats that were given FURO treatment using the single 18-24 h FURO protocol (ISO: OIL n = 6; EB, n = 6; FURO: OIL n = 7; EB, n = 6) or the multiple 18-24 h FURO protocol (ISO: OIL n = 7; EB, n = 7; FURO: OIL n = 7; EB, n = 7) as described (Figures 5 and 7).

*Termination, and Tissue Collection*

After a single 18-24 h or multiple 18-24 h treatment with FURO or ISO, rats were anesthetized with carbon dioxide ($CO_2$; 1.5 -2 L/min) and decapitated. Trunk blood was collected into heparinized tubes for analysis of plasma protein, sodium, and potassium concentration, with aliquots collected into micro-hematocrit capillary tubes for analysis of hematocrit as described in experiment 1. The brain was rapidly removed, frozen on dry ice, and stored at -80° C. Uteri were collected and analyzed as described in experiment 1

*Western Blot*

Frozen brains were removed from -80°C freezer and transferred into a cryostat at -12°C for blocking. A hindbrain section (~500 µm) containing the dorsal vagal complex was located by approximating stereotaxic coordinates according to Paxinos and Watson (Paxinos and Watson 1998). With the brain on its ventral surface, a single sided razor blade was used to cut the spinal cord at approximately Bregma -16 and across the primary fissure of the cerebellum between the $5^{th}$ and $6^{th}$ lobules at approximately Bregma -12. Tissue punches from the dorsal vagal complex were taken from hindbrain sections using micro-punches (<1mm I.D.). Punches were weighed and stored in Eppendorf tubes at -80°C. Tissue punches were homogenized on ice in triple-detergent lysis buffer (Vargas 2005) using a Sonic Dismembrator (Model 100) set at 1. Samples were centrifuged at 12,000 rpm for 30 minutes at 4°C and the supernatant transferred into new tubes. Protein concentration was determined using a bicinchoninic acid assay (BCA assay) where bovine serum albumin (BSA; 1mg/ml) was used for the standard curve preparation. The total protein concentrations were measured at 570 nm using a Bio Tek Synergy 2 microplate reader and Gen 5 1.11 imager software (BioTek Instruments, Inc., Winooski, VT, USA).

Equal amount of protein (20µg) from each sample was transferred into a micro-tube with 5X modified Laemmli buffer (pH 6.8) with loading dye (0.25 M Tris HCl; 10% sodium dodecyl sulfate; 10mM dithithritol; 30% glycerol; 0.05% bromphenol blue; 50 µL/mL β–mercaptoethanol) and centrifuged for 30s. Samples were denatured by boiling in a thermocycler (Simpli Amp) at 99.9°C for 10 minutes. Samples and protein ladder were electrophoresed on 12% acrylamide gels in 1X running buffer at 50V for 20 minutes and 110 volts for 2.5 hours.

Proteins were electrophoretically transferred at 100 volts for 50 minutes onto low fluorescent polyvinylidene difluoride (PVDF) membrane, pre-soaked in 20% methanol. Blots were blocked at room temperature for 2 hours in 5% bovine serum albumin (BSA)-1X Tris-buffered saline with

0.1 % Tween (TBST). After blocking, blots were rinsed with 1X TBST and incubated overnight at 4°C on a rocker in fresh 5% BSA-TBST containing one of the following commercially available primary antibodies: glial fibrillary acidic protein (Millipore mouse anti-GFAP; 1:6000), calcium calmodulin kinase (Thermo Fisher mouse anti CamKII; 1:1000), or β-actin (#4970; Cell Signaling rabbit anti-β-actin; 1:1000). The next day, blots were washed 3X for 10 minutes each with 1X TBST. Blots were incubated at 25°C on a rocker for 2 hours in secondary antibodies (Amersham ECL Plex goat anti-mouse or anti-rabbit IgG, Cy5; 1:2500 or Cy3; 1:2500) followed by additional washes with 1X TBST (6X for 10 minutes each).

*Image Analysis*

Blots were imaged using the GE Health Care Typhoon Scanner 9410. Semi-quantitative analysis of immunoblots were performed using freeware NIH ImageJ software (National Institutes of Health, Bethesda, MD) using β-actin as loading control.

**Statistical Analysis**

All data are presented as means ± S.E. Statistica software (StatSoft) was used for all analyses. The difference between means was considered to be significant when $P<0.05$; the magnitude of the difference between groups was assessed using effect size (partial eta squared, Ηp). Comparisons of significant main effects or interactions were made using two-tailed Fisher's least-significant difference (LSD) tests. For experiment 1, three-way ANOVA with hormone (OIL, EB), drug (ISO, FURO) and frequency (2 h, single 18-24 h) as factors was used to analyze GFAP-ir in rostral, middle, and caudal sections from the AP and in the caudal and middle sections from the NTS.

For experiment 2, the change in body weight in OIL or EB treated rats from day 3 to day 1, given multiple OIL/EB treatments was analyzed using a two-way ANOVA with hormone (OIL, EB) and week (week 1, week 2, week 3, week 4) as factors, repeated for week. Uterine weight normalized to body weight was analyzed using a t-test. Water and salt intake by OIL or EB treated rats after FURO/ISO was normalized to body weight by dividing the volume of intake by the body weight in 100 grams. Two-way ANOVA with hormone (OIL, EB) and week (week 1, week 2, week 3, week 4) as factors, repeated for week, was used to analyze the change in body weight after FURO/ISO treatment, urine volume, overnight urine volume, overnight urine sodium excretion, overnight urine potassium excretion, overnight urine osmolality, water, salt, and overnight water intake. Two-way ANOVA with hormone (OIL, EB) and week (week 2, week 3, week 4) as factors, repeated for week, was used to analyze urinary sodium excretion, urinary potassium excretion, urine osmolality, water balance, and sodium balance.

For experiment 3, the change in body weight in OIL or EB treated rats from day 3 to day 1, given multiple OIL/EB treatments was analyzed using a two-way ANOVA with hormone (OIL, EB) and week (week 1, week 2, week 3) as factors, repeated for week. Uterine weight normalized to body weight was analyzed using a t-test. Three-way ANOVA with hormone (OIL, EB), drug (ISO, FURO) and week (week 1, week 2, week 3) as factors, repeated for week, was used to analyze the change in body weight after FURO/ISO treatment and overnight water intake normalized to body weight. Three-way ANOVA with hormone (OIL, EB), drug (ISO, FURO), and week (week 0, week 1, week 2) as factors, repeated for week, was used to analyze water and salt intake normalized to body weight. Two-way ANOVA with hormone (OIL, EB) and drug (ISO, FURO) was used to analyze hematocrit, plasma protein, plasma sodium, plasma potassium, and plasma osmolality. The change in protein expression from control (ISO) of GFAP normalized to beta-actin expression was analyzed using a three-way ANOVA with hormone (OIL, EB), drug (ISO, FURO), and frequency (single 18-24 hr, multiple 18-24 hr) as factors. Finally, the change

in protein expression from control (ISO) of CaMKII normalized to beta-actin expression was analyzed using a two-way ANOVA with hormone (OIL, EB) and drug (ISO, FURO) as factors for the single 18-24 h FURO group.

# CHAPTER IV

# RESULTS

## Experiment 1: Furosemide Effects on Astrocytes in the Dorsal Vagal Complex

### *Area Postrema*

GFAP-ir in the area postrema (AP) (Figures 7-12) differed between FURO and ISO treated rats with the effect of hormone depending on level of AP.

Three-way ANOVA revealed a main effect of drug [$F(1,24)= 6.3$, $p<0.05$, $\eta_p^2 = 0.21$] on GFAP-ir in the caudal AP (Figure 8), with FURO decreasing GFAP-ir independent of hormone or protocol. There were no main effects of hormone or protocol, and no interactions.

Three-way ANOVA revealed a main effect of drug [$F(1,23)= 4.6$, $p<0.05$, $\eta_p^2 = 0.17$] on GFAP-ir in the middle AP (Figure 10), with FURO decreasing GFAP-ir independent of hormone or protocol. Here, too, there were no main effects of hormone or protocol, and no interactions.

Three-way ANOVA revealed a main effect of drug [$F(1,22) = 16.8$, $p<0.001$, $\eta_p^2 = 0.43$] on GFAP-ir in the rostral AP (Figure 12), with FURO decreasing GFAP-ir independent of hormone or protocol. There were no main effects of hormone or protocol, and no interactions.

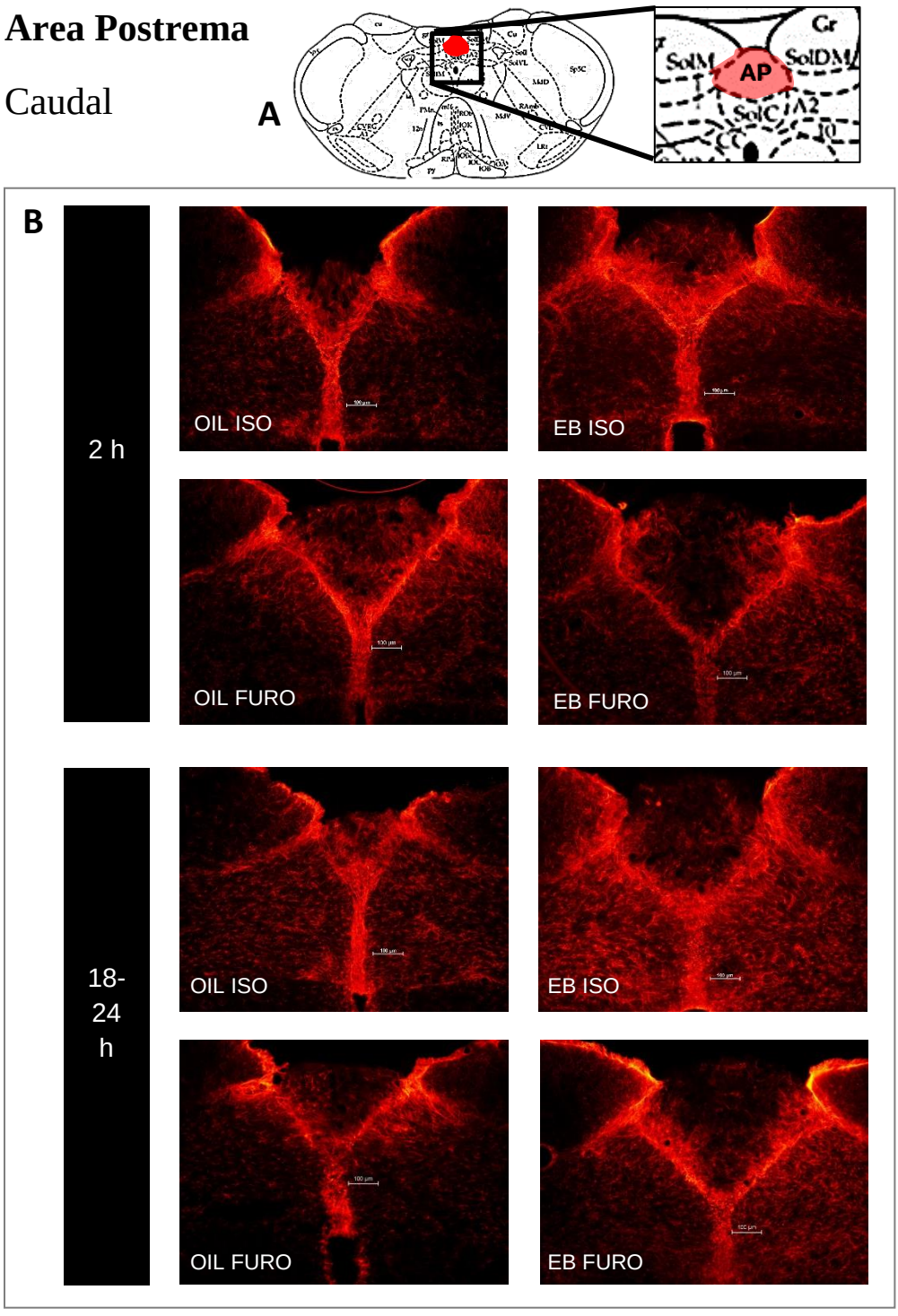


Figure 8. **Schematic and representative photomicrographs of GFAP immunoreactivity (GFAP-ir) in the caudal area postrema (AP).** (A) Schematic representation of a coronal section through the rat hindbrain highlighting AP (red shading); adapted from Paxinos and Watson 1998. Area outlined in black is enlarged to the right. (B) Representative fluorescent photomicrographs of GFAP (red staining) in the caudal AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) as described. Rate were given s.c. injections of 0.15 M NaCl (ISO) or furosemide (FURO) and then terminated 2 hours or 18-24 hours later. Scale bar, 100 µm.

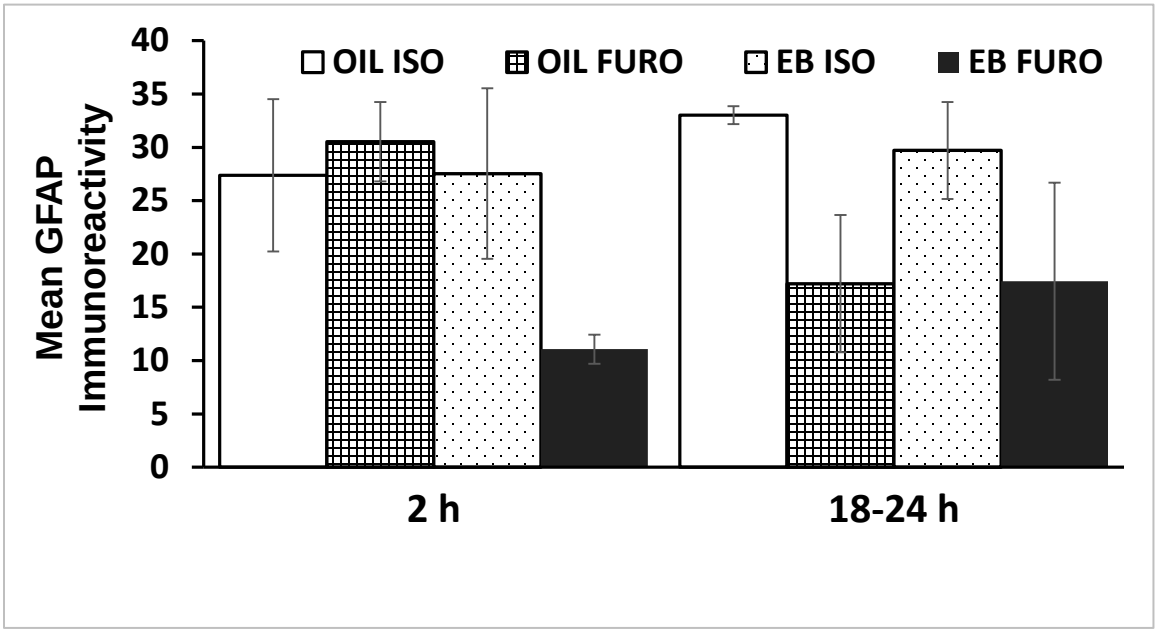


Figure 9. **Mean GFAP immunoreactivity (GFAP-ir) in the caudal area postrema (AP).** GFAP-ir in the caudal AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) treatment and s.c. injections of 0.15 M NaCl (ISO: OIL, white bars; EB, dotted bars) or furosemide (FURO: OIL, grid bars; EB, black bars) and terminated 2 hours or 18-24 hours later. There was a significant effect of drug, with GFAP-ir in FURO treated rats less than that in ISO treated rats.

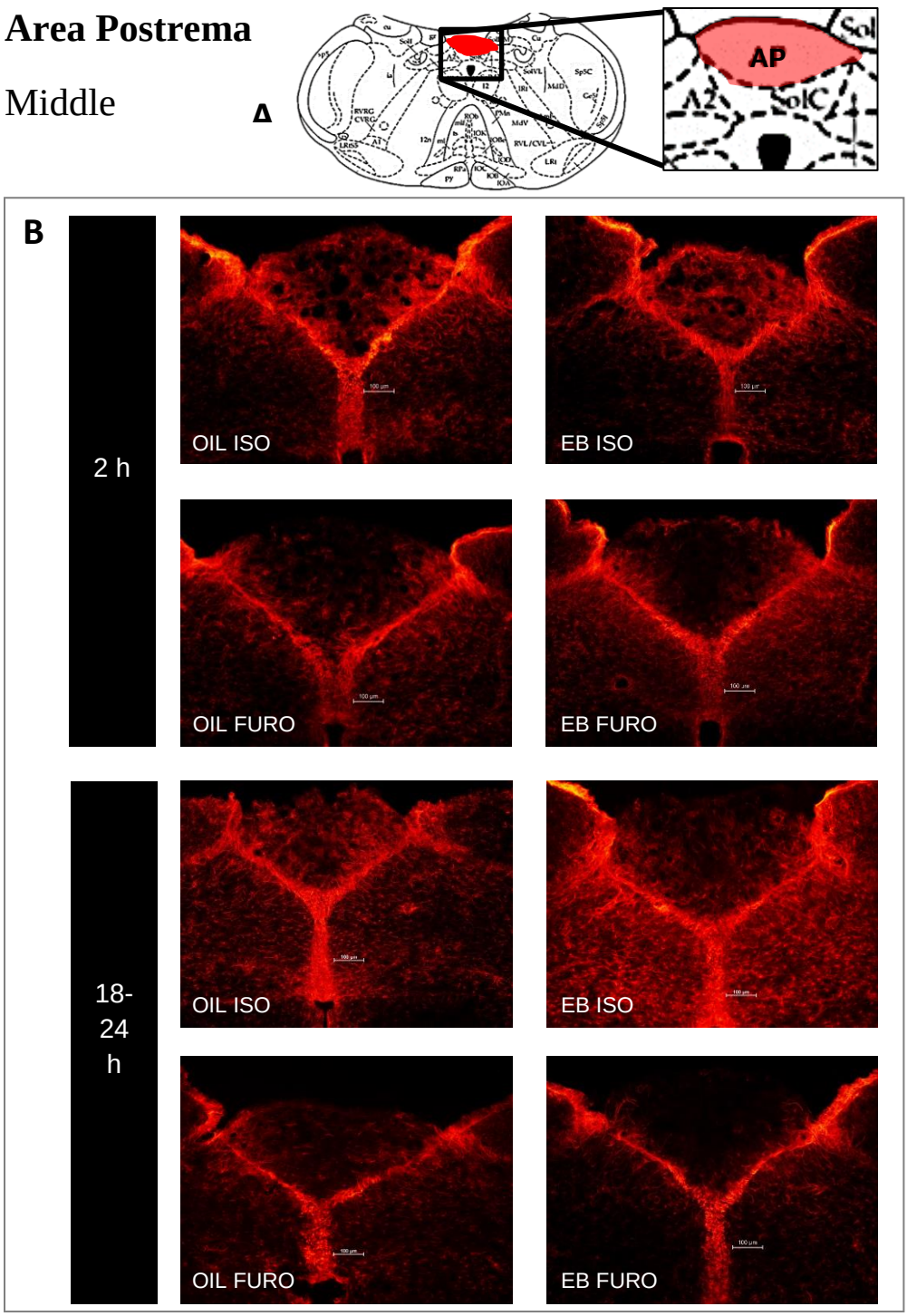


Figure 10**. Schematic and representative photomicrographs of GFAP immunoreactivity (GFAP-ir) in the middle area postrema (AP).** (A) Schematic representation of a coronal section through the rat hindbrain highlighting AP (red shading) adapted from Paxinos and Watson 1998. Area outlined in black is enlarged to the right. (B) Representative fluorescent photomicrographs of GFAP (red staining) in the middle AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO) or furosemide (FURO) and then terminated 2 hours or 18-24 hours later. Scale bar, 100 µm.

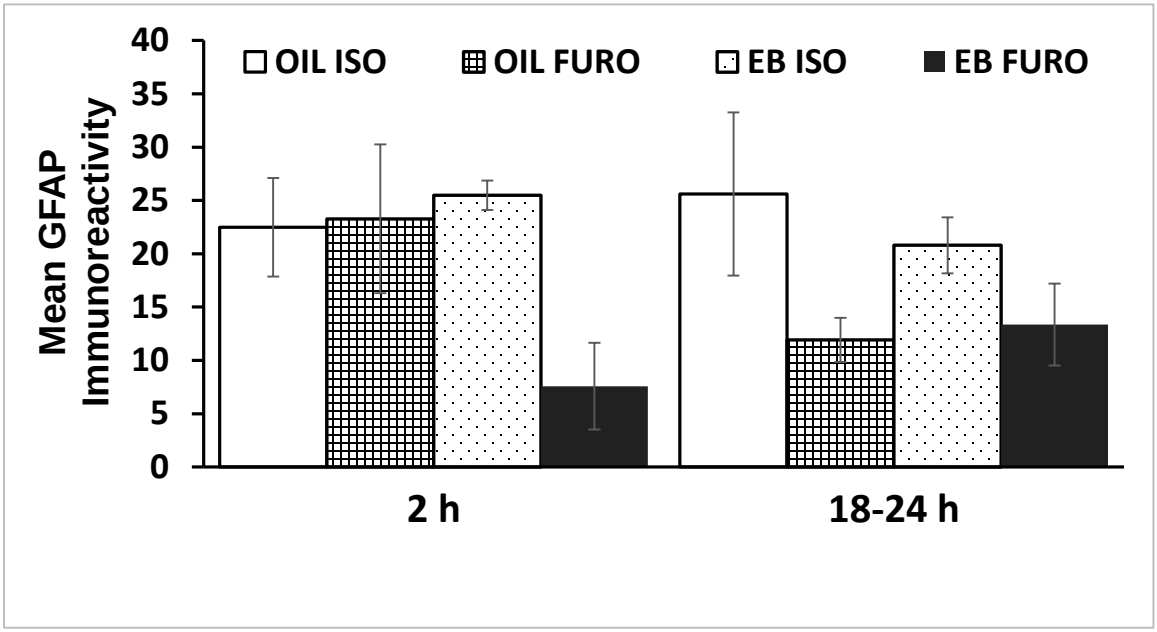


Figure 11. **Mean GFAP immunoreactivity (GFAP-ir) in the middle area postrema (AP).** GFAP-ir in the middle AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO: OIL, white bars; EB, dotted bars) or furosemide (FURO: OIL, grid bars; EB, black bars) and terminated 2 hours or 18-24 hours later. There was a significant effect of drug, with GFAP-ir in FURO treated rats less than that in ISO treated rats.

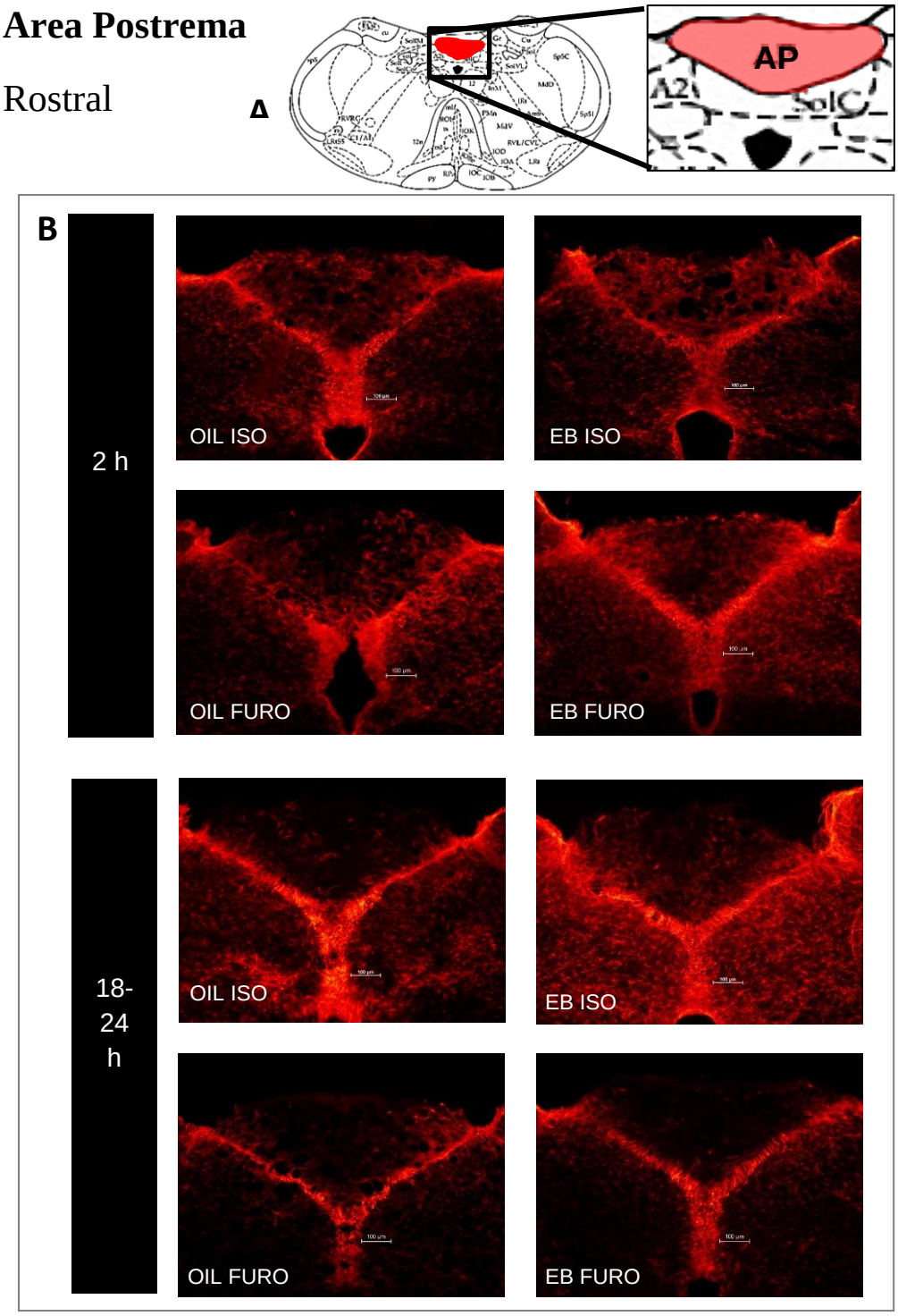


Figure 12. **Schematic and representative photomicrographs of GFAP- immunoreactivity (GFAP-ir) in the rostral area postrema (AP).** (A) Schematic representation of a coronal section through the rat hindbrain highlighting AP (red shading) adapted from Paxinos and Watson 1998. Area outlined in black is enlarged to the right. (B) Representative fluorescent photomicrographs of GFAP (red staining) in the rostral AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO) or furosemide (FURO) and then terminated 2 hours or 18-24 hours later. Scale bar, 100 µm.

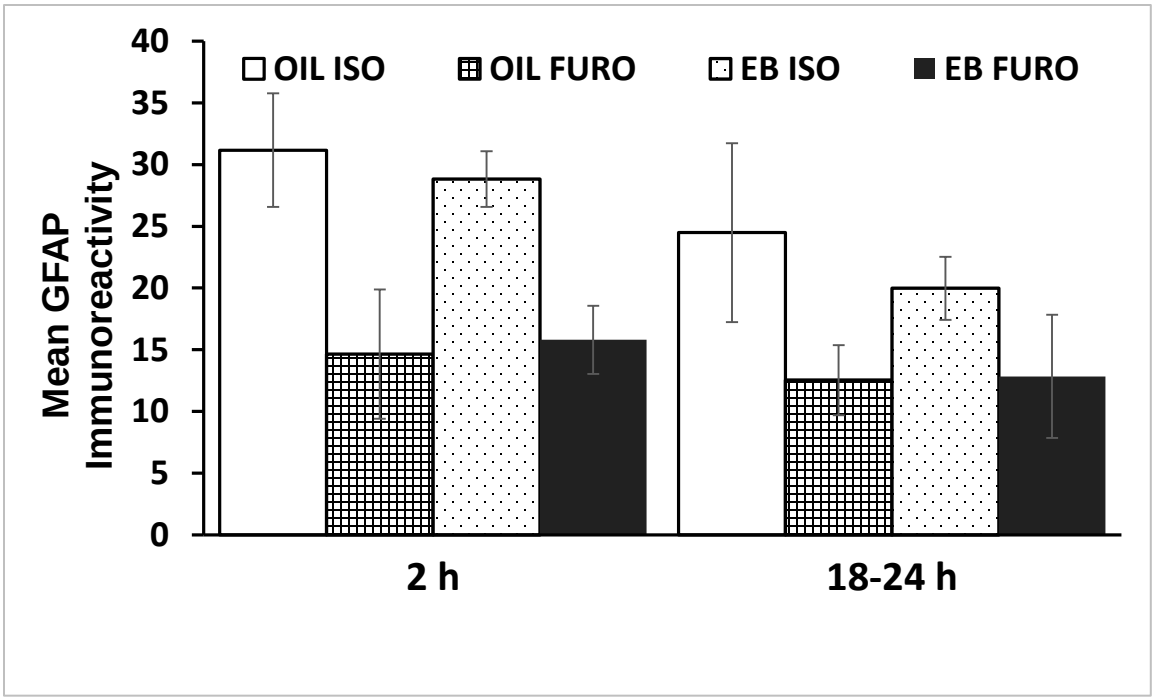


Figure 13. **Mean GFAP immunoreactivity (GFAP-ir) in the rostral area postrema (AP).** GFAP-ir in the rostral AP of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO: OIL, white bars; EB, dotted bars) or furosemide (FURO: OIL, grid bars; EB, black bars) and then terminated 2 hours or 18-24 hours later. There was a significant effect of drug, with GFAP-ir in FURO treated rats less than that in ISO treated rats.

*Nucleus of the Solitary Tract*

Three-way ANOVA revealed a main effect of protocol [$F(1,32) = 5.5$, $p<0.05$, $\eta_p^2 = 0.15$] on GFAP-ir in the caudal NTS (Figure 14), as well as a protocol by drug interaction [$F(1,32) = 13.6$, $p<0.001$, $\eta_p^2 = 0.30$]. Comparisons of this interaction revealed GFAP-ir was greater in the 18-24 h FURO protocol (ps< 0.05, 0.001, 0.05, 0.01). There were no main effects of hormone or drug and no other interactions.

Three-way ANOVA revealed a main effect of drug [$F(1,28) = 5.4$, $p<0.05$, $\eta_p^2 = 0.16$] on GFAP-ir in the middle NTS (Figure 16), as well as a protocol by drug interaction [$F(1,28) = 12.4$, $p<0.01$, $\eta_p^2 = 0.31$]. Comparisons of this interaction revealed GFAP-ir was greater in the 18-24 h FURO protocol (ps< 0.05, 0.01, 0.01). There were no main effects of hormone or protocol and no other interactions.

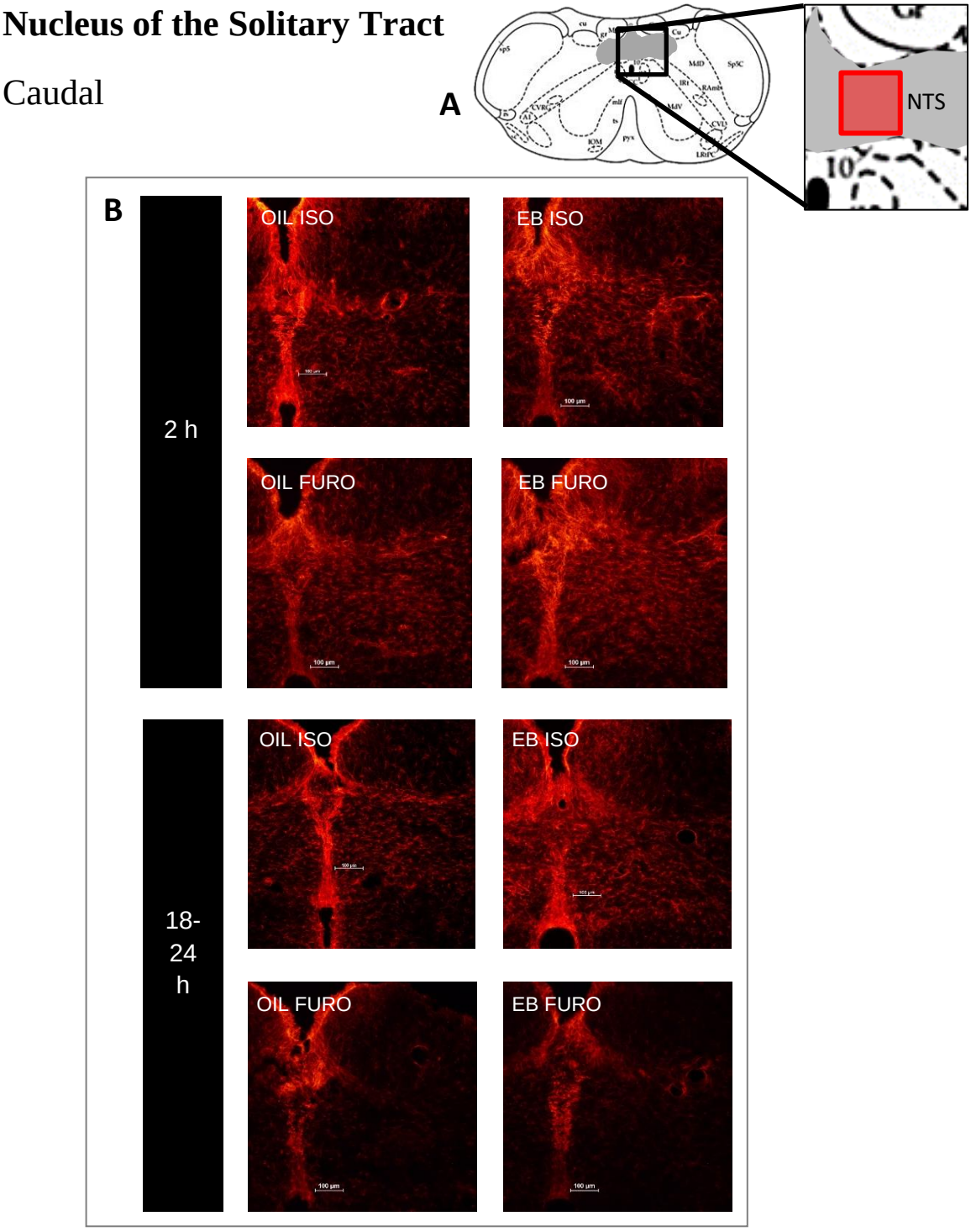


Figure 14. **Schematic and representative photomicrographs of GFAP immunoreactivity (GFAP-ir) in the caudal nucleus of the solitary tract (NTS).** (A) Schematic representation of a coronal section through the rat hindbrain highlighting the caudal NTS (grey shading) adapted from Paxinos and Watson 1998. Area outlined in black is enlarged to the right; red shading indicates the area from which measurements were taken. (B) Representative fluorescent photomicrographs of GFAP (red staining) in the caudal NTS of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO) or furosemide (FURO) and then terminated 2 hours or 18-24 hours later. Scale bar, 100 µm.

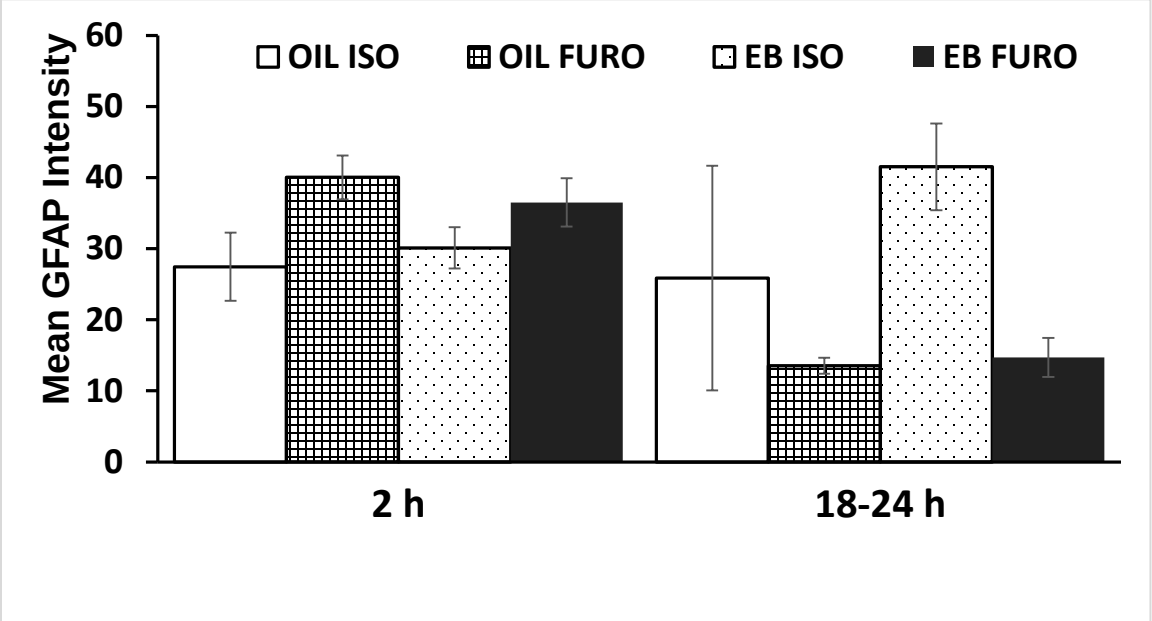


Figure 15. **Mean GFAP immunoreactivity (GFAP-ir) in the caudal nucleus of the solitary tract (NTS).** GFAP-ir in the caudal NTS of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO: OIL, white bars; EB, dotted bars) or furosemide (FURO: OIL, grid bars; EB, black bars) and then terminated 2 hours or 18-24 hours later. There was a significant effect of protocol and a protocol by drug interaction.

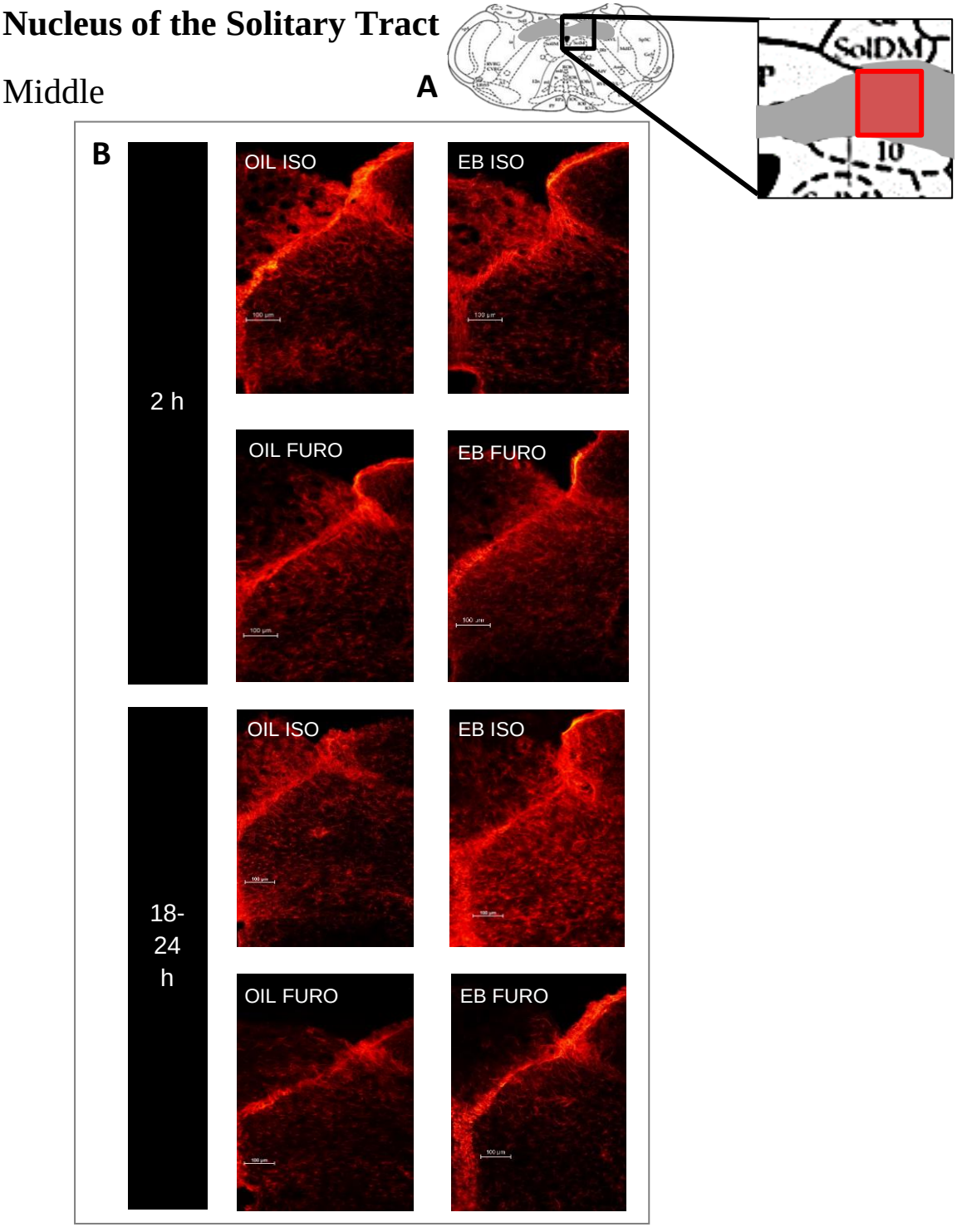


Figure 16. **Schematic and representative photomicrographs of GFAP immunoreactivity (GFAP-ir) in the middle nucleus of the solitary tract (NTS).** (A) Schematic representation of a coronal section through the rat hindbrain highlighting the middle NTS (grey shading) adapted from Paxinos and Watson 1998. Area outlined in black is enlarged to the right; red shading indicates the area from which measurements were taken. (B) Representative fluorescent photomicrographs of GFAP (red staining) in the middle NTS of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO) or furosemide (FURO) and then terminated 2 hours or 18-24 hours later. Scale bar, 100 µm.

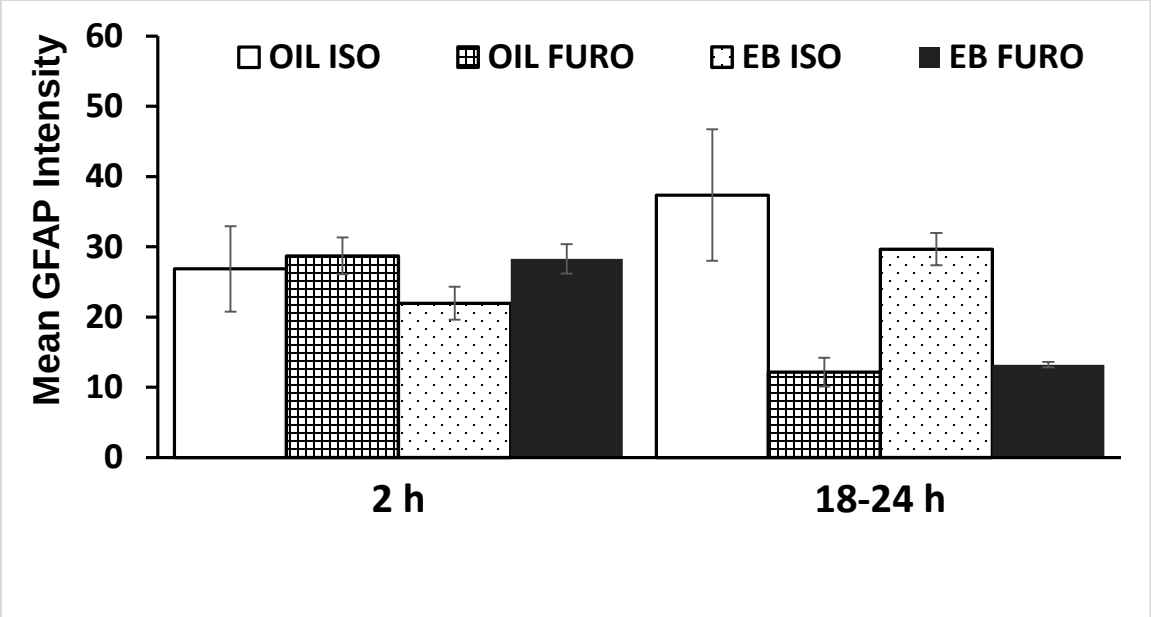


Figure 17. **Mean GFAP immunoreactivity (GFAP-ir) in the middle nucleus of the solitary tract (NTS).** GFAP-ir in the middle NTS of OVX rats given OIL vehicle (OIL) or estradiol benzoate (EB) and s.c. injections of 0.15 M NaCl (ISO: OIL, white bars; EB, dotted bars) or furosemide (FURO: OIL, grid bars; EB, black bars) and then terminated 2 hours or 18-24 hours later. There was a significant effect of drug and a drug by protocol interaction.

**Experiment 2: Furosemide Effects on Measures of Fluid Balance and Behavioral Responses**

*Efficacy of Recurrent Estrodial Benzoate Treatment*

Body weight (Figure 17) of ovariectomized rats differed between OIL- and EB-treated rats during the hormone regimen prior to drug treatment. Two-way rm-ANOVA revealed a main effect of hormone [$F(1,7) = 7.6$, $p<0.05$, $\eta_p^2 = 0.52$] on the change in body weight from day 3 to day 1 but no main effect of time or interactions.

Uterine weight normalized to body weight (Figure 18) in OVX rats was significantly greater after EB treatment compared to that after OIL treatment ($t = -7.86$; $p<0.001$).

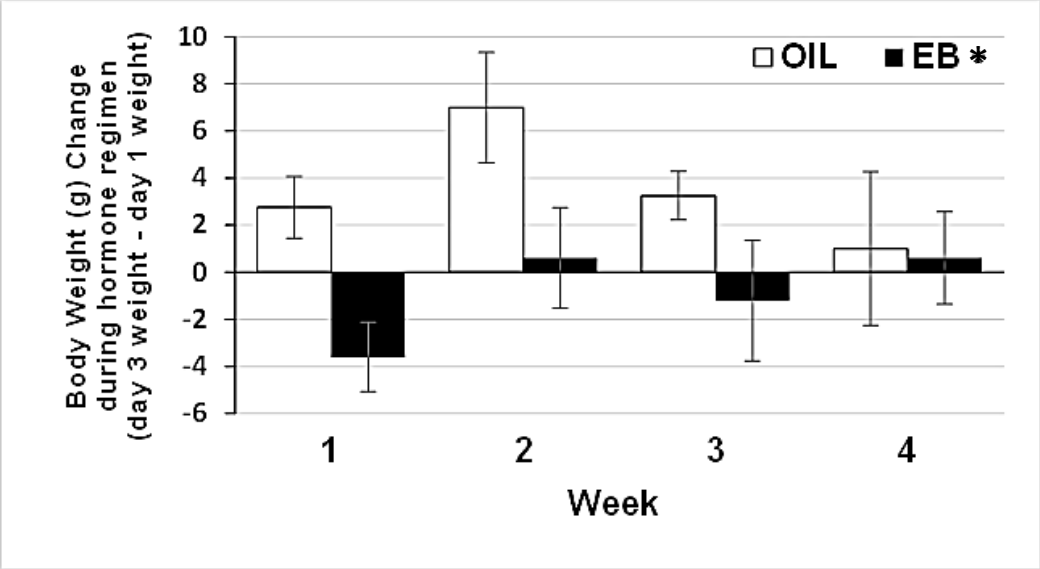


Figure 18. **Efficacy of recurrent estradiol benzoate (EB) treatment on body weight:** Change in body weight (g) during the hormone treatment regimen in OIL vehicle (OIL; white bars; n=4) treated and estradiol benzoate-treated (EB; black bars; n=5) OVX rats prior to injection with FURO (FURO) or 0.15 M NaCl (ISO) * = significantly different from OIL.

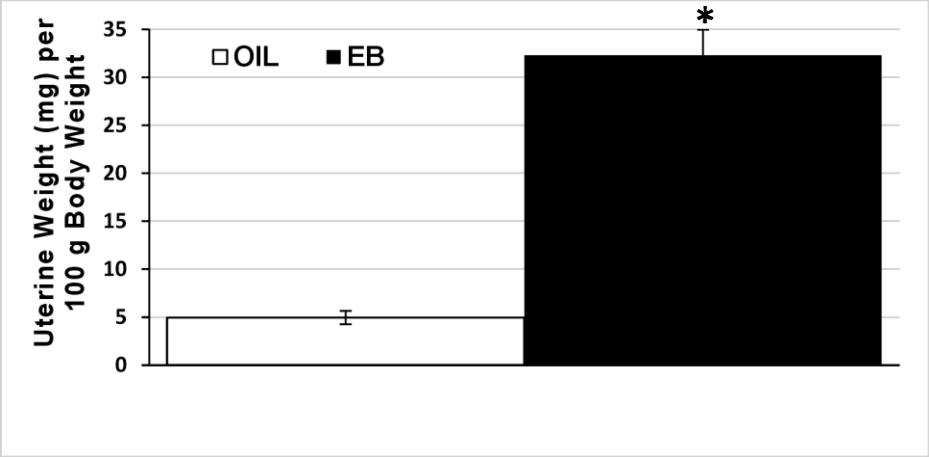


Figure 19. **Efficacy of estradiol benzoate (EB) treatment on uterine weight:** Uterine weight (mg) in OIL vehicle-treated (OIL; white bar) and estradiol benzoate-treated (EB; black bar) OVX rats sacrificed 18-24 hours after injection with FURO (FURO) or 0.15 M NaCl (ISO) on day 4. * = significantly different from OIL.

*Efficacy of Multiple Furosemide Treatments*

Two-way rm-ANOVA revealed a main effect of time [$F(1,3) = 134.1$; $p<0.001$, $\eta_p^2 = 0.95$] and no hormone by time interaction on body weight loss (Figure 19) in OVX rats. Comparisons of the body weight loss over time revealed that, independent of hormone treatment, weight loss in week one (the ISO treatment week) was significantly less ($ps<0.001$) than that in all other weeks (the FURO treatment weeks). Body weight loss during the FURO treatment weeks were not different from each other.

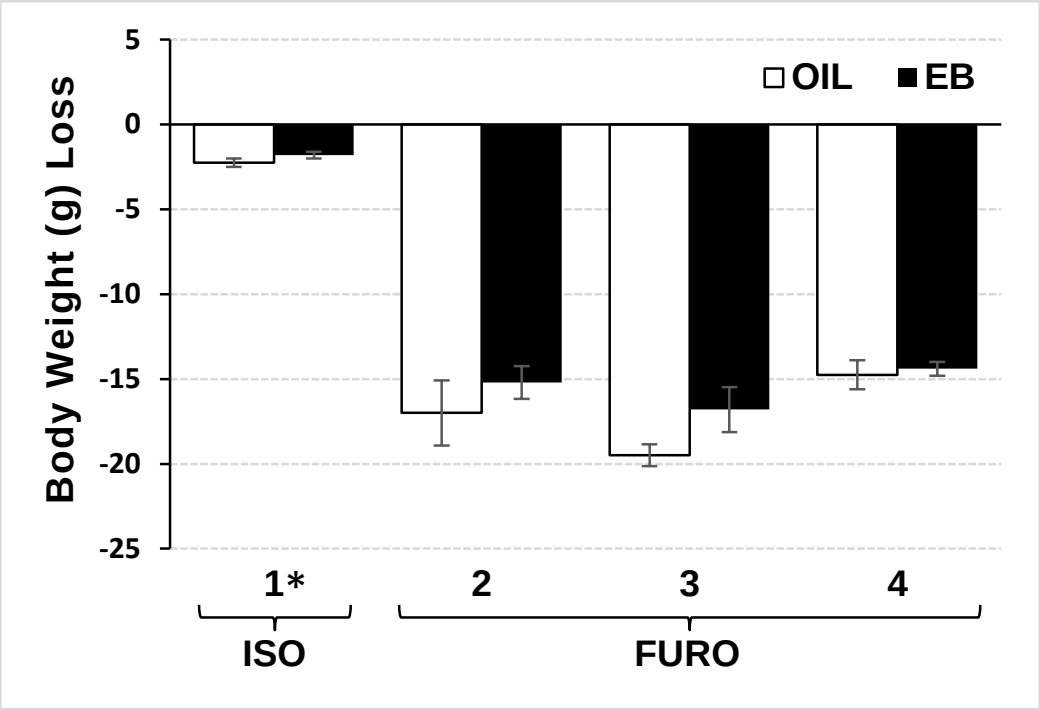


Figure 20. **Efficacy of multiple furosemide (FURO) treatments on body weight:** Body weight loss by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats two hours after injection with furosemide (FURO) or 0.15 M NaCl (ISO) treatment in a within subjects design using the multiple 18-24 h protocol. Within the hormone groups, each rat served as its own control, receiving ISO treatment for one week and then FURO treatment weekly for three weeks. * = significantly less from all other weeks; ps<0.001.

*Urinary Excretion*

In rats given multiple FURO treatments in a within subjects design, two-way rm-ANOVA revealed a main effect of week [F(1,3) = 198.2; p<0.001, $\eta_p^2$ = 0.97] on urine volume (Figure 20). Pairwise comparisons of the main effect of week revealed that urine volume in week 1, the week of ISO treatment, was significantly less than that in weeks 2-4, the FURO treatment weeks (ps< 0.001). Urine volume, and therefore urine sodium and potassium excretion, were negligible after ISO. Accordingly, analyses of urinary sodium and potassium excretion and urine osmolality were conducted only after FURO treatment (i.e., weeks 2-4). Two-way rm-ANOVA revealed a main effect of week on urinary sodium excretion (Figure 21) [F (2, 14) = 11.8, p<0.001, $\eta_p^2$ = 0.63],

urinary potassium excretion (Figure 22) [F (2, 14) = 9.0, p<0.01, $\eta_p^2$ = 0.56], and urine osmolality (Figure 0) [F (2, 14) = 5.3, p<0.05, $\eta_p^2$ = 0.43]. Planned comparisons revealed that sodium excretion (ps<0.001-0.05) and potassium excretion (ps<0.01-0.05) was significantly greater on week 3, the second week of furosemide treatment compared to other weeks. In all cases, pairwise comparisons revealed no effects of hormone, nor were there interactions between hormone and week.

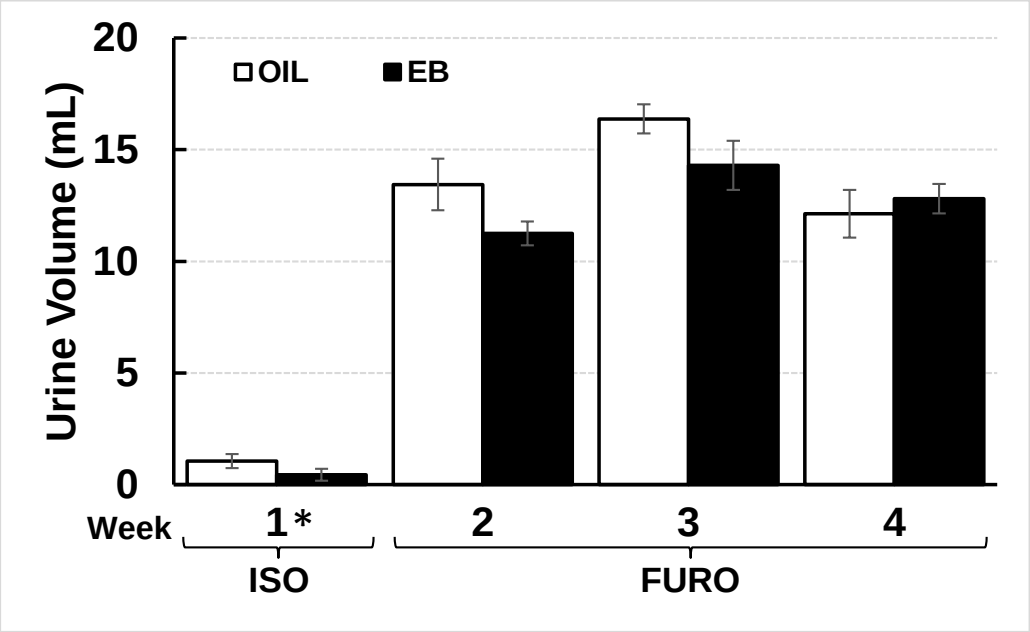


Figure 21. **Urine volume:** Urine output (mL) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats two hours after FURO and ISO treatment in a within subjects design. Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1 and FURO treatment on week 2-4. * = significantly less than all other weeks (ps<0.001).

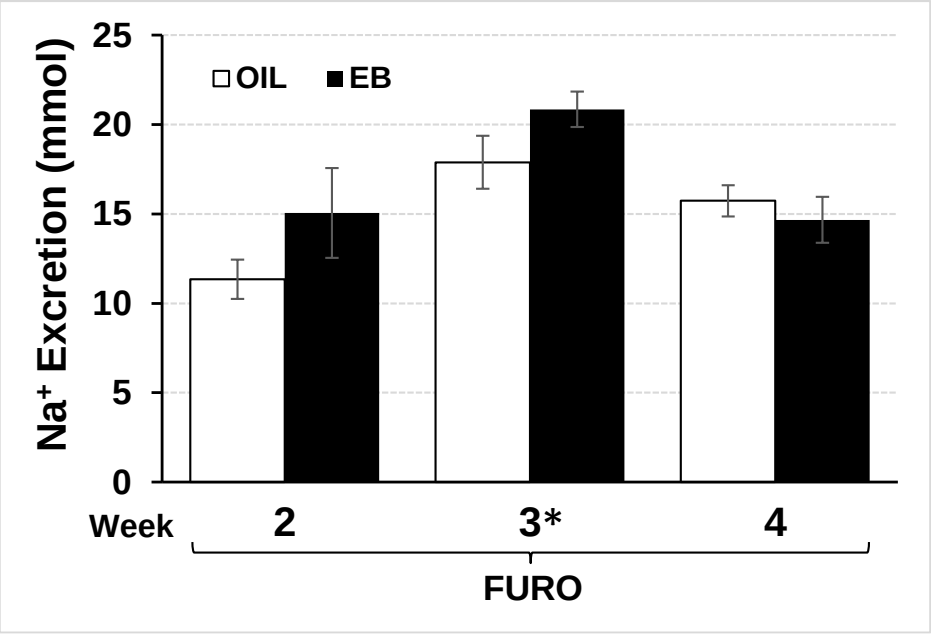


Figure 22. **Urine sodium ($Na^+$) excretion:** Urine $Na^+$ excretion by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats two hours after FURO treatment in a within subjects design. Within the hormone groups, each rat served as its own control. Sodium excretion was significantly greater on week 3, the second week of furosemide treatment compared to other weeks (ps<0.001-0.05). Urine volume, and therefore urine sodium and potassium excretion, were negligible after ISO. Accordingly, analyses of urinary sodium and potassium excretion and urine osmolality were conducted only after FURO treatment (i.e., weeks 2-4).

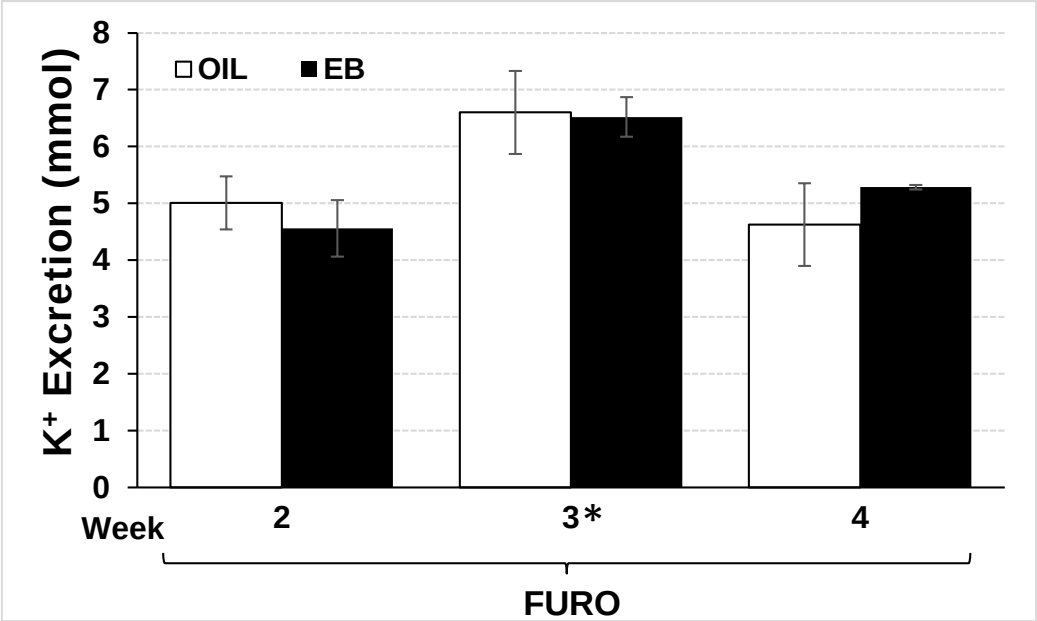


Figure 23. **Urine potassium ($K^+$) excretion:** Urine $K^+$ excretion by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats two hours after FURO treatment in a within subjects design. Within the hormone groups, each rat served as its own control. Potassium excretion was significantly greater on week 3, the second week of furosemide treatment compared to other weeks (ps<0.01-0.05). Urine volume, and therefore urine sodium and potassium excretion, were negligible after ISO. Accordingly, analyses of urinary sodium and potassium excretion and urine osmolality were conducted only after FURO treatment (i.e., weeks 2-4).

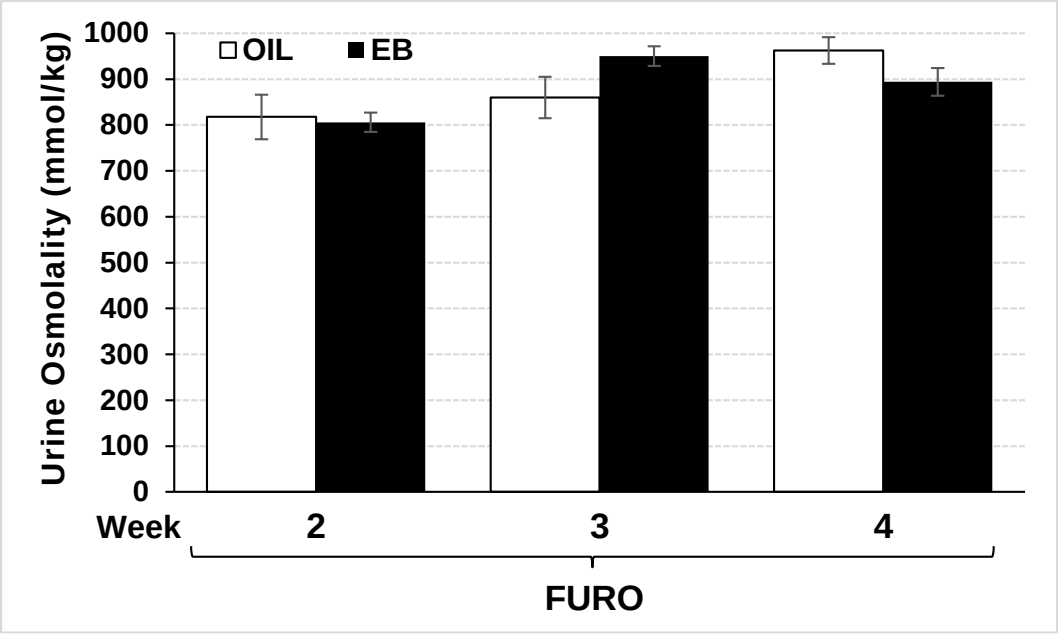


Figure 24. **Urine osmolality:** Urine osmolality (mmol/kg) in OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats two hours after FURO treatment in a within subjects design. Within the hormone groups, each rat served as its own control. Urine volume, and therefore urine sodium and potassium excretion, were negligible after ISO. Accordingly, analyses of urinary sodium and potassium excretion and urine osmolality were conducted only after FURO treatment (i.e., weeks 2-4).

*Overnight Urinary Excretion*

Two-way rm-ANOVA revealed no main effects or interactions for overnight urine volume, sodium excretion, potassium excretion, or urine osmolality (Figures 24-27).

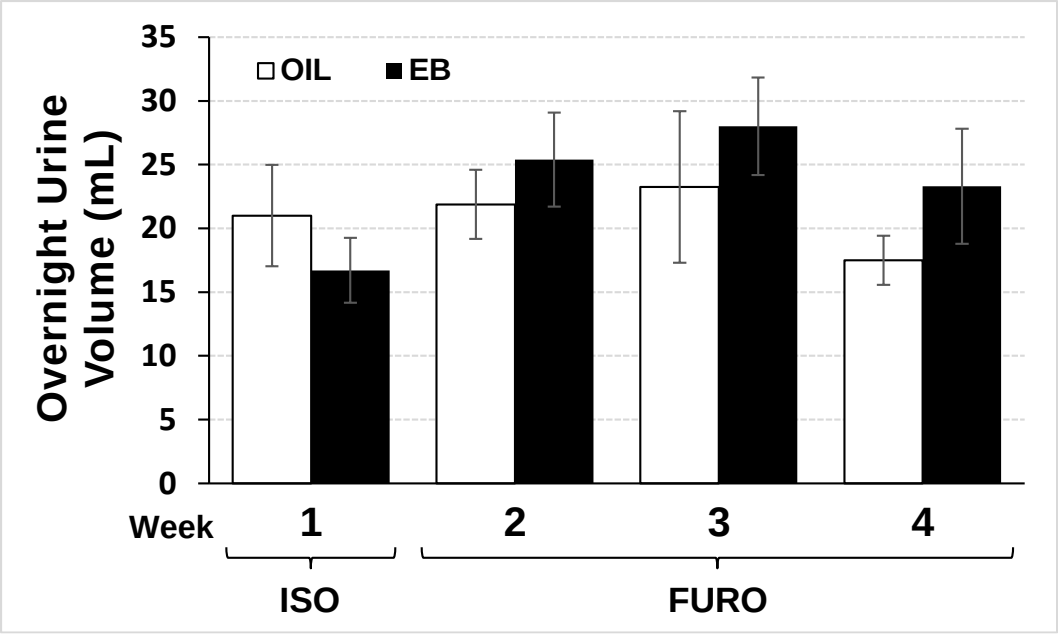


Figure 25. **Overnight urine volume:** Overnight urine output (mL) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO or ISO treatment in a within subjects design using the multiple 18-24 h protocol. Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1and FURO treatment on week 2-4. Urine was collected 18-24 hour after injection. There were no main effects or interactions.

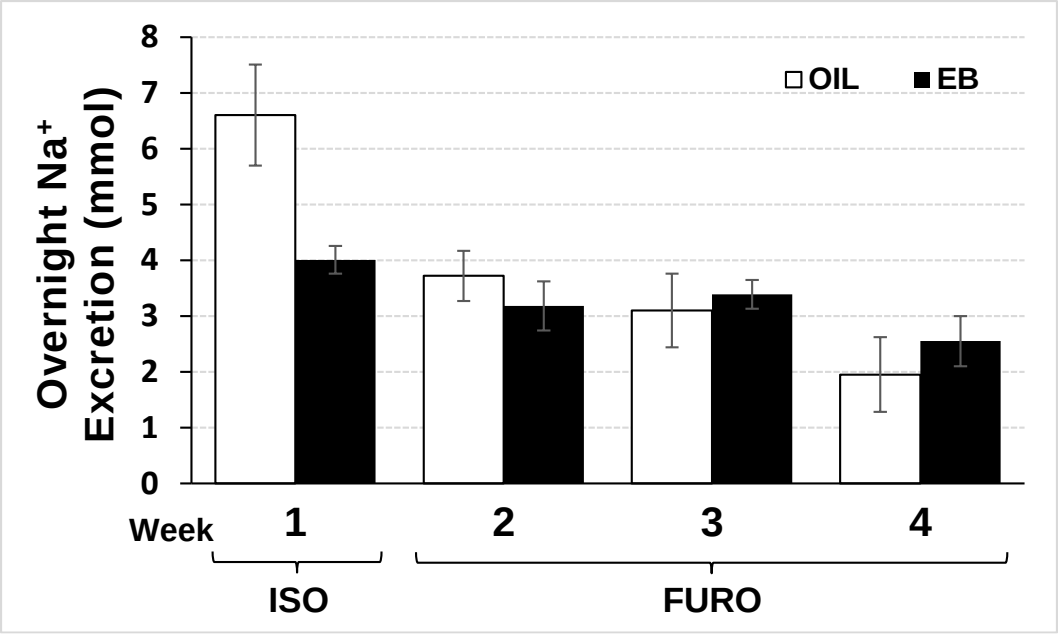


Figure 26. **Overnight sodium ($Na^+$) excretion:** Overnight urine $Na^+$ excretion (mmol) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO or ISO treatment in a within subjects design using the multiple 18-24 h protocol. Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1and FURO treatment on week 2-4. Urine was collected 18-24 hour after injection. There were no main effects or interactions.

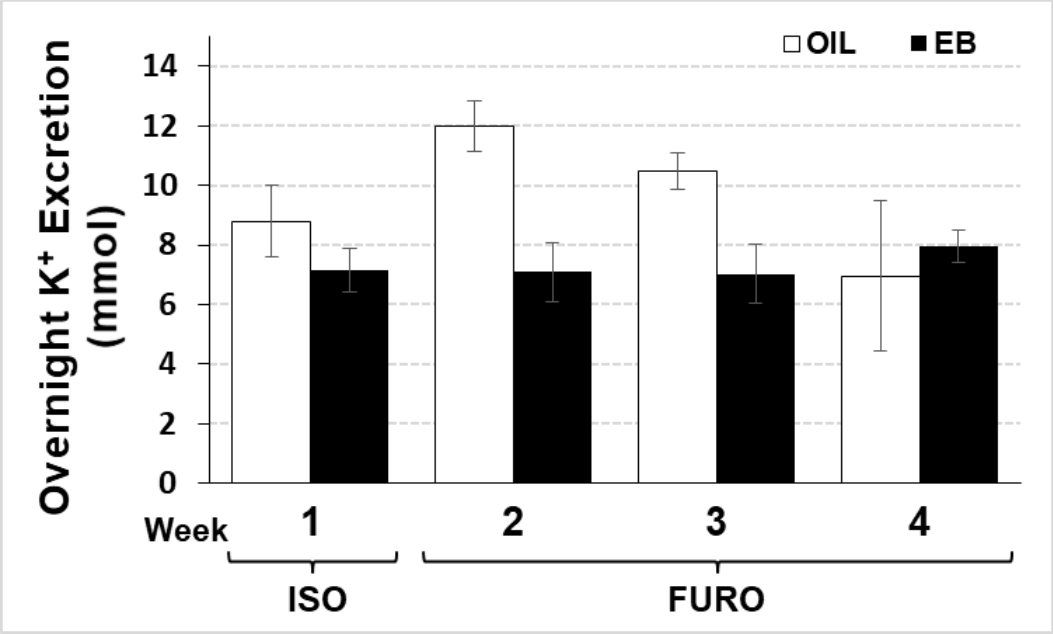


Figure 27. **Overnight potassium ($K^+$) excretion:** Overnight urine $K^+$ excretion (mmol) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO or ISO treatment in a within subjects design using the multiple 18-24 h protocol. Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1and FURO treatment on week 2-4. Urine was collected 18-24 hour after injection. There were no main effects or interactions.

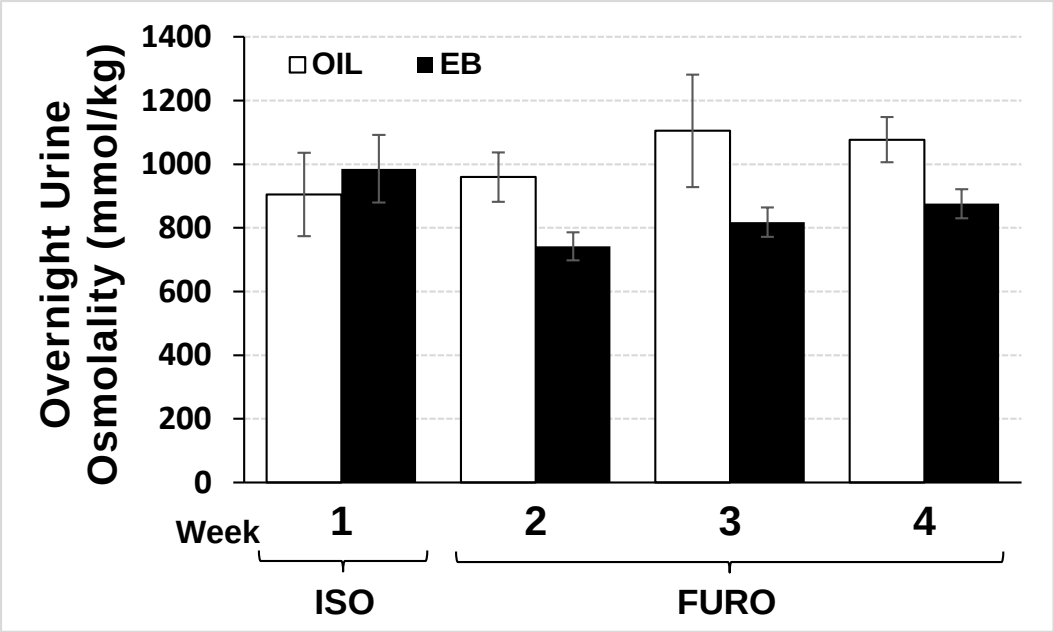


Figure 28. **Overnight urine osmolality:** Overnight urine osmolality (mmol/kg) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO or ISO treatment in a within subjects design using the multiple 18-24 h protocol. Within the hormone groups, each rat served as its own control, receiving ISO treatment on week 1and FURO treatment on week 2-4. Urine was collected 18-24 hour after injection. There were no main effects or interactions.

*Overnight Water Intake*

In OIL or EB treated rats given multiple FURO treatments in a within subjects design, two-way rm-ANOVA revealed a main effect of week [F (3, 21) = 6.1, p<0.01, $\eta_p^2$ = 0.46] on overnight water intake normalized to body weight (Figure 28). Pairwise comparisons of overnight water intake over time revealed that water intake was significantly less (ps<0.01-0.05) on week 1 compared to week 2-4 (FURO treatment). Overnight water intake on the FURO treatment weeks were not different from each other. There was no main effect of hormone and no interactions.

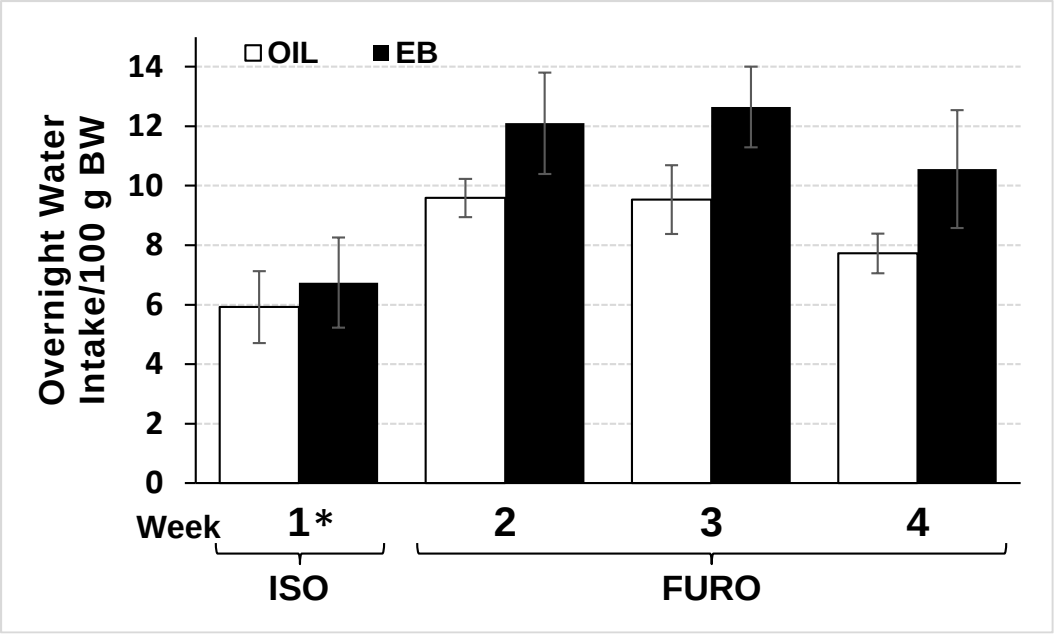


Figure 29. **Overnight water intake:** Overnight water intake/100 g body weight (BW) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO and ISO treatment in a within subjects design using the multiple 18-24 h protocol. * = Overnight water intake on week 1 was significantly less than that on week 2, 3, 4.

*Sodium and Water Balance*

Two-way rm-ANOVA revealed a significant main effect of week [$F(2,14) = 9.2$, $p<0.01$, $\eta_p^2 = 0.57$] on sodium balance (Figure 29). Pairwise comparisons revealed a greater decrement in sodium balance on week 3 (ps<0.01-0.05) compared to weeks two or four. There were no effects for the calculated water balance (Figure 30).

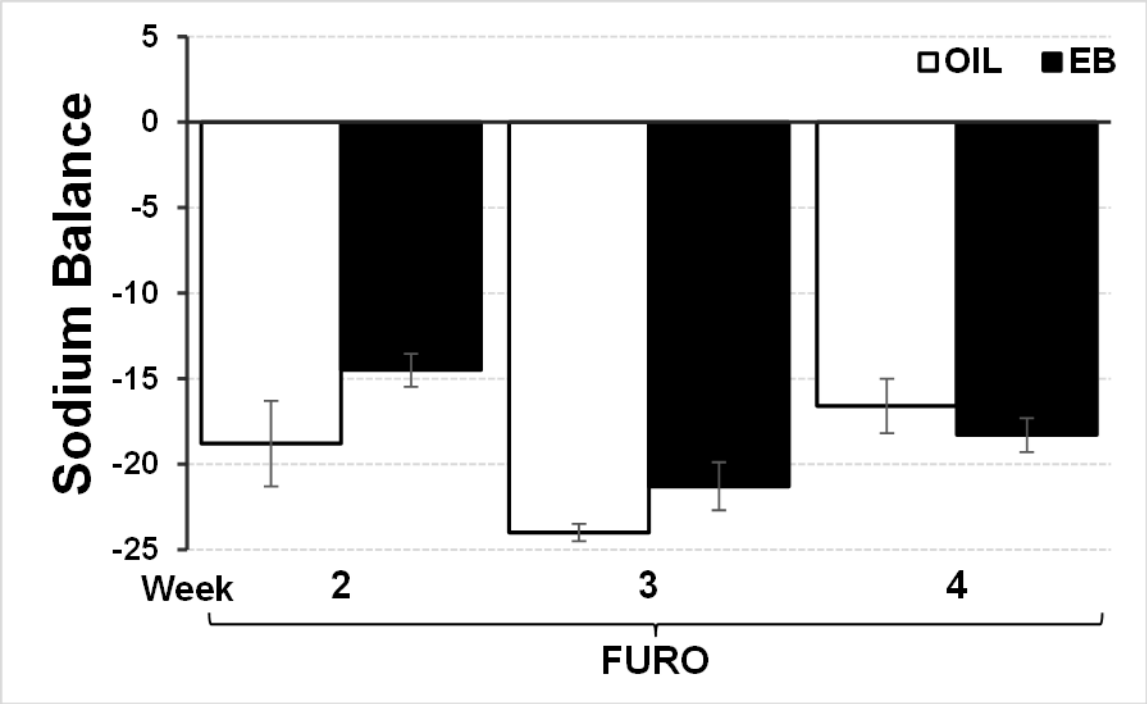


Figure 30. **Sodium balance:** Sodium balance (intake-output) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO treatment in a within subjects design using the multiple 18-24 h protocol. * = Sodium balance on week 3 was significantly less than that on week 2 and 4.

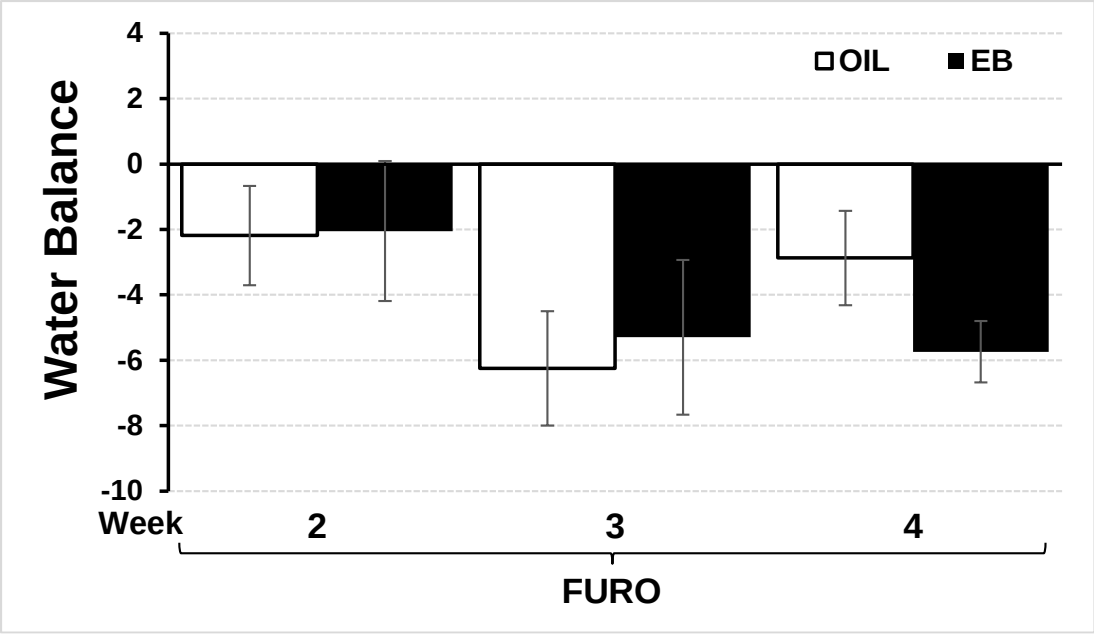


Figure 31. **Water balance:** Water balance (intake-output) by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats given FURO treatment in a within subjects design using the multiple 18-24 h protocol. There were no main effects or interactions.

*2 h Salt Intake*

In OIL- or EB-treated rats given multiple FURO treatments in a within subjects design, two-way rm-ANOVA revealed a significant main effect of week [F (3, 18) = 17.7, p<0.001, $\eta_p^2$ = 0.75] on salt intake (Figure 31) normalized to body weight, but no main effect of hormone or interactions. Planned comparisons revealed that independent of hormone, salt intake on week 1 (the ISO week) was less than that on weeks 2, 3, or 4 (the FURO weeks) by OIL- (ps<0.05, 0.001, 0.01) and EB- (ps<0.01, 0.001, 0.001) treated rats given multiple FURO treatments in a within subjects design.

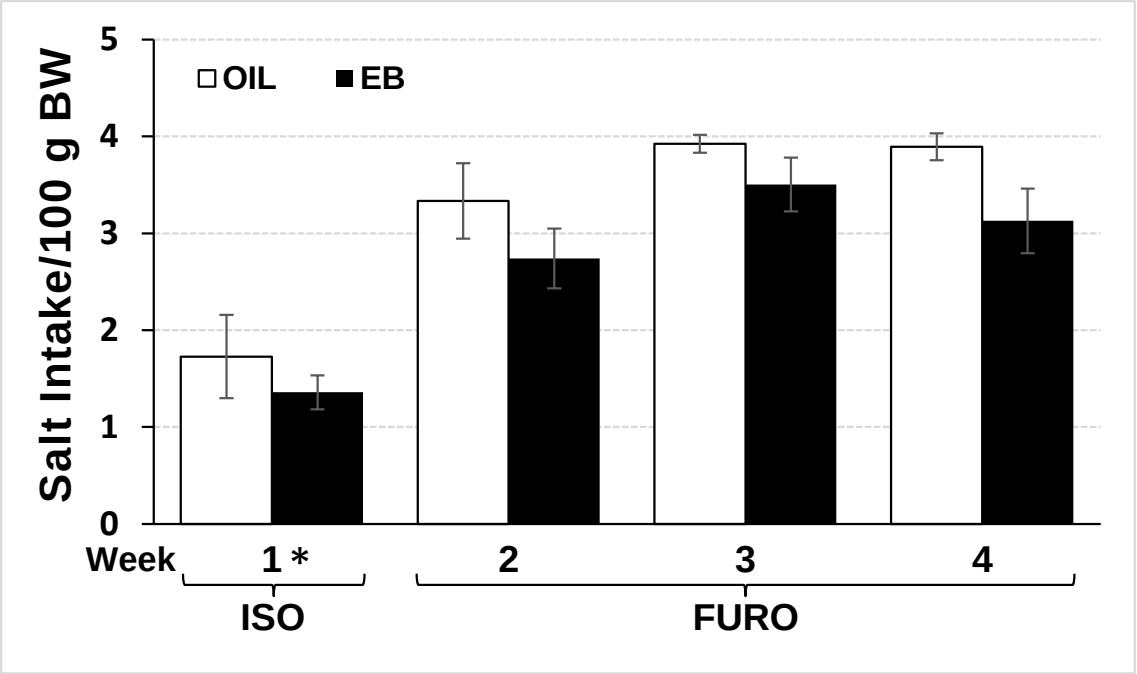


Figure 32. **2 h Salt Intake:** Salt intake/100 g body weight (BW) during a two hour test by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats. Testing began on day 4, 18-24 hour after the second injection with 0.15 M NaCl (ISO) or furosemide (FURO) in a within-subjects design using the multiple 18-24 h protocol. * = Salt intake on week 1 was significantly less than that on week 2-4.

*2 h Water Intake*

In OIL or EB treated rats given multiple FURO treatments in a within subjects design, two-way rm-ANOVA revealed a main effect of week [F (3, 21) = 7.3, p<0.01, $\eta_p^2$ = 0.51] on water intake normalized to body weight (Figure 32), no main effect of hormone, or interactions. Planned comparisons of the main effect of week revealed that rats drank less water during week 1 (the ISO week) compared to weeks 2, 3, and 4 (the FURO weeks) (EB: ps<0.01, 0.05, 0.01; OIL: ps<0.05).

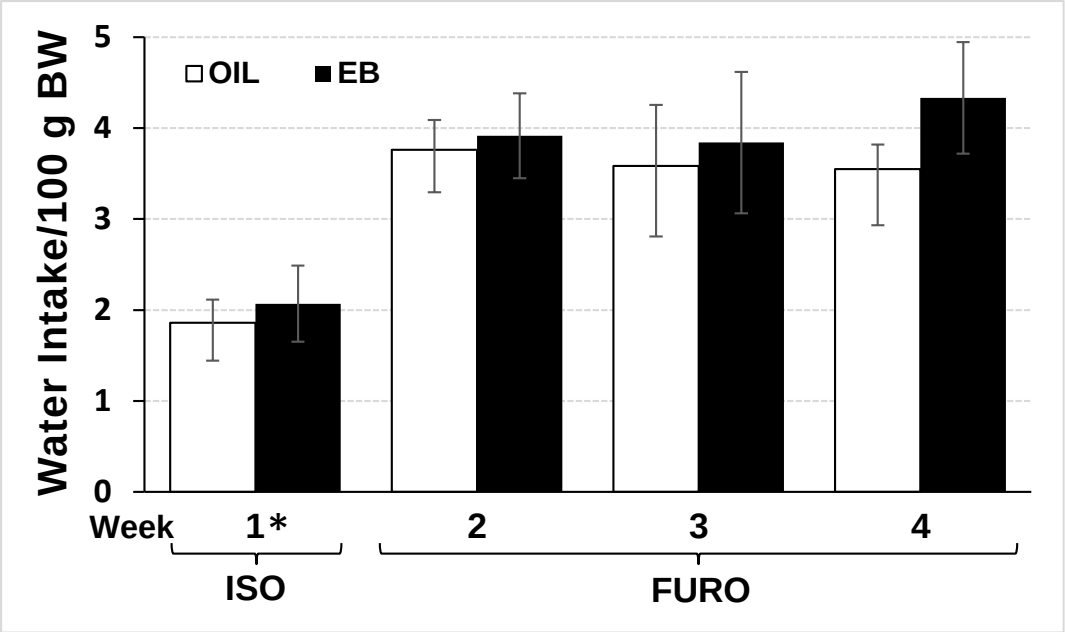


Figure 33. **2 h Water intake:** Water intake/100 g body weight (BW) during a two hour test by OIL- (white bars; n = 4) and EB- (black bars; n = 5) treated rats. Testing began on day 4, 18-24 hour after the second injection with 0.15 M NaCl (ISO) or furosemide (FURO) in a within-subjects design using the multiple 18-24 h protocol. * = Water intake on week 1 was significantly less than that on week 2-4.

**Experiment 3: Furosemide Effects on Synaptic Proteins**

Single 18-24 Hour Furosemide

*Efficacy of Estrodial Benzoate Treatment*

The change in body weight (Table 1) of OVX rats differed between OIL and EB treated rats over the hormone regimen prior to drug treatment, and was significantly less in OVX rats after EB treatment compared to that in OIL-treated rats ($t = 10.48$; $p<0.001$). Uterine weight (Table 1) in OVX rats was significantly greater after EB treatment compared to that in OIL-treated rats ($t = -12.4$; $p<0.001$).

Table 1. **Efficacy of estradiol benzoate (EB) treatment.** Change in body weight (g) during the hormone treatment regimen (day 3 weight-day 1 weight) in oil vehicle (OIL; n=15) treated and estradiol benzoate-treated (EB; n=12) OVX rats prior to injection with a single 18-24 h furosemide (FURO) or 0.15 M NaCl (ISO). Uterine weights (mg/100 g body weight) were assessed at sacrifice on day 4, twenty-four hours after a single 18-24 h FURO or ISO. * = significantly different from OIL.

| | **Change in body weight (g)** | **Uterine weight (mg/100 g body weight)** |
|---|---|---|
| **OIL** | 8.4 ± 0.8 | 10.6 ± 0.4 |
| **EB** | -5.3 ± 1.0* | 27.8 ± 1.4* |

*Efficacy of a Single Furosemide Treatment*

Body weight loss (Table 2) differed in OVX rats given FURO or ISO. Two-way ANOVA revealed a main effect of drug [$F(1,23) = 178.0$; $p<0.001$, $\eta_p^2 = 0.89$] on the change in body weight

with greater weight loss 2 hours after FURO. There was no main effect of hormone and no interaction.

There was no effect of hormone or drug on plasma sodium concentration in OVX rats (Table 2), and no interaction between factors.

Two- way ANOVA revealed a main effect of drug on plasma protein concentration [$F(1,23) = 17.7$; $p<0.001$, $\eta_p^2 = 0.43$], hematocrit [$F(1,23) = 13.5$; $p<0.01$, $\eta_p^2 = 0.37$], plasma potassium concentration [$F(1,23) = 10.7$; $p<0.01$, $\eta_p^2 = 0.32$], and plasma osmolality [$F(1,23) = 5.7$; $p<0.05$, $\eta_p^2 = 0.20$] assessed on day 4, twenty four hours after FURO or ISO, with greater plasma protein, hematocrit, and plasma osmolality (Table 2) after FURO independent of hormone. Plasma potassium concentration (Table 2) was decreased after FURO. There was no effect of hormone and no interaction for any of these measures.

Table 2. **Efficacy of furosemide after a single 18-24 h treatment.** Body weight loss (g) in oil vehicle-treated (OIL; n=6-9/condition) and estradiol benzoate-treated (EB; n=6/condition) OVX rats two hours after a single 18-24 h furosemide (FURO) or 0.15 M NaCl (ISO) treatment. Plasma protein concentration (pPro; g/dL), hematocrit, plasma $Na^+$ concentration ($pNa^+$; mMol/L plasma water), plasma $K^+$ concentration ($pK^+$; mMol/L plasma water), and plasma osmolality (mmol/kg) were assessed at sacrifice on day 4, twenty four hours after FURO or ISO. # = significantly different from ISO.

| | **Body weight loss** | | **pPro** | | **Hematocrit** | |
|---|---|---|---|---|---|---|
| | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** |
| **OIL** | -5.5 + 1.1 | -17.6 + 0.6 | 6.5 + 0.2 | 6.9 + 0.1 | 45.9 + 1.3 | 51.7 + 0.8 |
| **EB** | -6.0 + 0.7 | -15.0 + 0.5 | 6.5 + 0.1 | 7.3 + 0.2 | 48.1 + 0.7 | 50.0 + 1.0 |
| | **$pNa^+$** | | **$pK^+$** | | **pOsm** | |
| | ***ISO*** | ***FURO*** | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** |
| **OIL** | 147.0 + 0.5 | 142.4 + 1.6 | 5.5 + 0.2 | 4.7 + 0.2 | 371.3 + 9.2 | 405.1 + 11.2 |
| **EB** | 146.6 + 1.4 | 146.2 + 0.7 | 5.3 + 0.4 | 4.3 + 0.1 | 376.8 + 13.6 | 415.0 + 20.1 |

Multiple 18-24 Hour Furosemide

*Efficacy of Recurrent Estrodial Benzoate Treatment*

Body weight change (Figure 33) in OVX rats prior to drug treatment differed between OIL- and EB-treated rats throughout the protocol. Two-way rm-ANOVA revealed a main effect of hormone [$F(1,52) = 80.4$; $p<0.001$, $\eta_p^2 = 0.76$] on the change in body weight, with OIL-treated rats gaining weight and EB-treated rats losing weight. There was no main effect of week and no interactions.

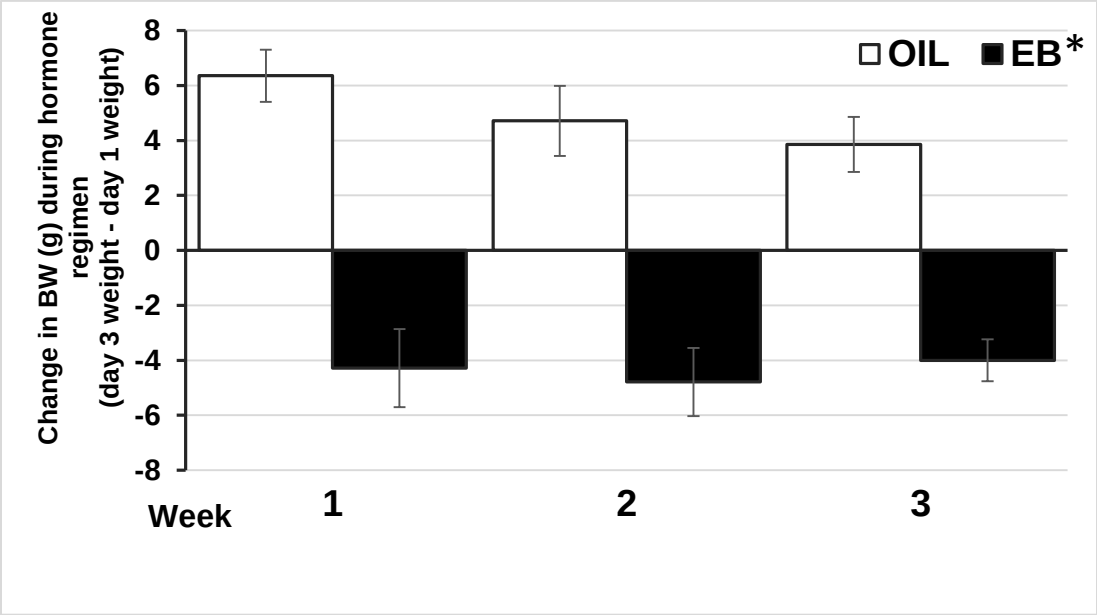


Figure 34. **Efficacy of recurrent estradiol benzoate (EB) treatment on body weight:** Change in body weight (g) during the hormone treatment regimen in OIL vehicle (OIL; white bars; n=14) treated and estradiol benzoate-treated (EB; black bars; n=14) OVX rats prior to injection with furosemide (FURO) or 0.15 M NaCl (ISO). * = significantly different from OIL.

Uterine weight (Figure 34) in OVX rats was significantly greater after EB treatment compared to that in OIL-treated rats ($t = -10.77$; $p<0.0001$).


Figure 35. **Efficacy of estradiol benzoate (EB) treatment on uterine weight:** Uterine weight (mg) per 100 g body weight (BW) in OIL vehicle-treated (OIL; white bar) and estradiol benzoate-treated (EB; black bar) OVX rats sacrificed on week 3, 18-24 hours after injection with furosemide (FURO) or 0.15 M NaCl (ISO). * = significantly different from OIL.

*Efficacy of Multiple Furosemide Treatment*

Three-way rm-ANOVA revealed a main effect of hormone [F(1,24) = 7.5; p<0.05, $\eta_p^2$= 0.24], a main effect of drug [F(1,24) = 507.7; p<0.001, $\eta_p^2$= 0.95], and a hormone by week interaction [F(1,48) = 4.3; p<0.05, $\eta_p^2$= 0.15] on body weight loss (Figure 35) two hours after injection with FURO or ISO treatment using the multiple 18-24 h protocol. Pairwise comparisons of the interaction revealed greater weight loss after FURO in OIL-treated rats compared to EB-treated rats during weeks 1 and 2 (ps<0.001-0.05) that did not persist in week 3.

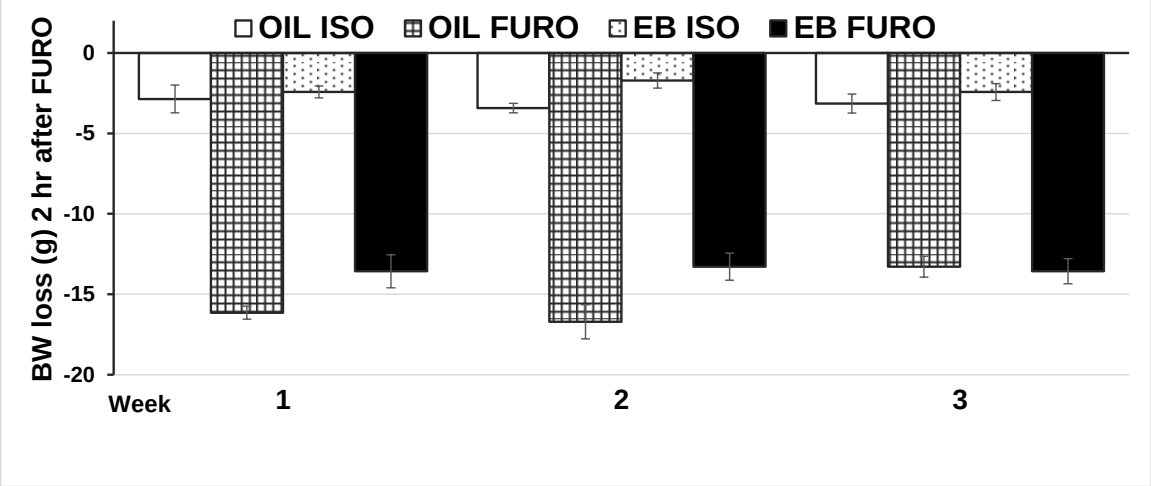


Figure 36. **Efficacy of multiple furosemide (FURO) treatments on body weight:** Body weight loss by OIL- (ISO: white bars, n = 7; FURO: grid bars, n=7) and EB- (ISO: dotted bars, n=7; FURO: black bars, n = 7) treated rats two hours after injection with FURO or 0.15 M NaCl (ISO) using the multiple 18-24 h protocol. There was a significant effect of drug, with greater weight loss after FURO than that after ISO and a significant effect of hormone, with greater weight loss in OIL-treated rats, independent of drug or week. Finally, there was a hormone by week interaction, with significantly greater weight loss in OIL- treated rats compared to EB-treated rats during weeks 1-2. This difference did not persist in week 3.

The average body weight loss over weeks 1-3 also was calculated to facilitate comparisons with the single 18-24 h FURO and multiple 18-24 h FURO protocols (Table 2, Table 3). Two-way ANOVA of the average body weight loss revealed a main effect of hormone [$F(1,24) = 7.5$; $p<0.05$, $\eta_p^2 = 0.24$] and a main effect of drug [$F(1,24) = 507.7$; $p<0.001$, $\eta_p^2 = 0.95$], with greater weight loss after FURO, but no main effect of hormone and no interaction.

Two- way ANOVA revealed a main effect of hormone on plasma sodium concentration [$F(1,20) = 5.5$; $p<0.05$, $\eta_p^2 = 0.22$], plasma protein concentration [$F(1,20) = 19.1$; $p<0.001$, $\eta_p^2 = 0.49$], and hematocrit [$F(1,24) = 13.5$; $p<0.01$, $\eta_p^2 = 0.36$] assessed at sacrifice on day 4, twenty four hours after the final FURO or ISO treatment (Table 3). EB-treated rats had greater plasma sodium and plasma protein concentrations independent of drug treatment, while hematocrit was less in EB-treated rats independent of drug treatment. There was no effect of hormone or drug on plasma potassium concentration or plasma osmolality in OVX rats, and no interaction between factors.

Table 3. **Efficacy of furosemide after multiple treatments.** Body weight loss (g) in oil vehicle-treated (OIL; n=6-9/condition) and estradiol benzoate-treated (EB; n=6/condition) OVX rats two hours after injection with a single 18-24 h furosemide (FURO) or 0.15 M NaCl (ISO) treatment. Plasma protein concentration (pPro; g/dL), hematocrit, plasma $Na^+$ concentration ($pNa^+$; mMol/L plasma water), plasma $K^+$ concentration ($pK^+$; mMol/L plasma water), and plasma osmolality (mmol/kg) were assessed at sacrifice on day 4, twenty four hours after 3rd FURO or ISO. # = significantly different from ISO.

| | **Body weight loss** | | **pPro** | | **Hematocrit** | |
|---|---|---|---|---|---|---|
| | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** |
| **OIL** | -3.1 + 0.4 | -15.4 + 0.5 | 6.9 + 0.1 | 6.9 + 0.1 | 54.8 + 1.1 | 54.4 + 1.7 |
| **EB** | -2.2 + 0.3 | -13.5 + 0.6 | 7.6 + 0.2 | 8.2 + 0.3 | 47.8 + 0.7 | 51.1 + 1.5 |
| | **$pNa^+$** | | **$pK^+$** | | **pOsm** | |
| | ***ISO*** | ***FURO*** | ***ISO*** | ***FURO#*** | ***ISO*** | ***FURO#*** |
| **OIL** | 146.3 + 1.9 | 140.3 + 1.1 | 7.9 + 0.9 | 6.7 + 0.8 | 397.5 + 16.8 | 362.8 + 8.2 |
| **EB** | 152.9 + 4.8 | 151.2 + 4.3 | 6.6 + 0.6 | 5.8 + 0.2 | 355.8 + 18.1 | 361.8 + 12.4 |

*Overnight Water Intake*

Three-way rm-ANOVA on overnight water intake (Figure 36) revealed a main effect of drug [F (1, 22) = 11.6, p<0.01, $\eta_p^2$ = 0.34] by OIL or EB treated rats given multiple FURO or ISO treatments, and no main effects of hormone or time, nor interactions.

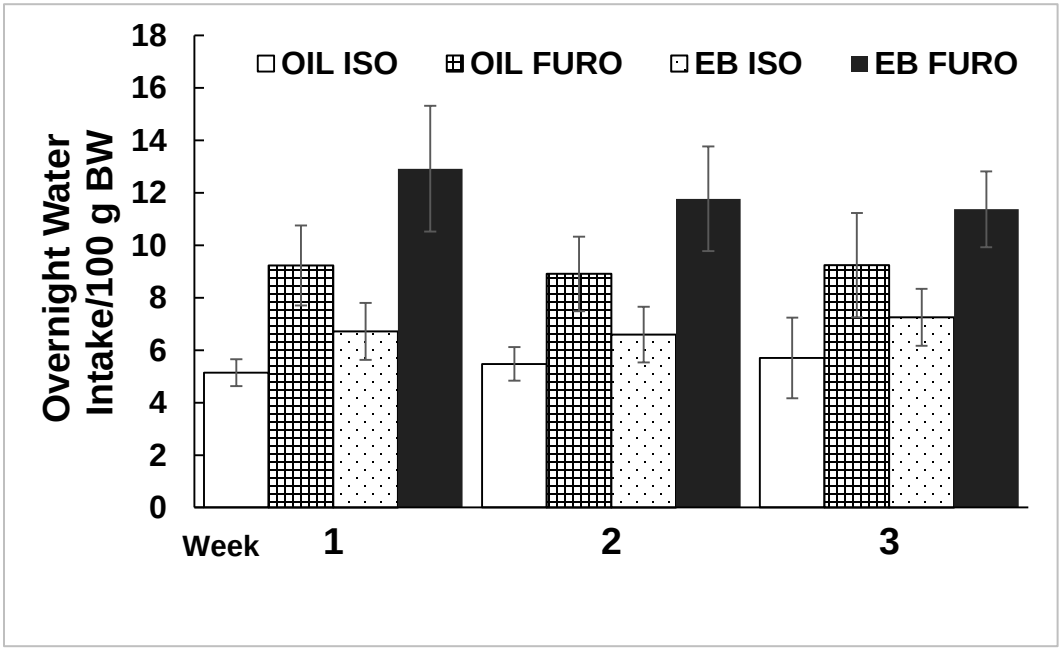


Figure 37. **Overnight water intake:** Overnight water intake/100 g body weight (BW) by OIL- (ISO: white bars, n = 7; FURO: grid bars, n=7) and EB- (ISO: dotted bars, n=7; FURO: black bars, n = 7) treated rats given FURO and ISO treatment using the multiple 18-24 h protocol. * = Overnight water intake on week 1 was significantly less than that on week 2, 3, 4.

*Water intake*

Three-way repeated measures ANOVA revealed a main effect of drug [F (1, 24) = 17.2, p<0.001, $\eta_p^2$ = 0.42], a main effect of time [F (2, 48) = 10.5, p<0.001, $\eta_p^2$ = 0.31], and a time by drug interaction [F (2, 48) = 8.4, p<0.001, $\eta_p^2$ = 0.26] for water intake normalized to bodyweight (Figure 37) by OIL or EB treated rats given multiple FURO or ISO treatments. Pairwise comparisons of the interaction revealed water intake by OIL treated rats given multiple FURO treatments increased after each weekly test (ps<0.05) except for the third test week. Water intake by EB treated rats given multiple FURO treatments increased after each weekly test (ps<0.01, 0.001, 0.05) and the water intake after the third test week was greater than that by OIL treated rats of the same week.

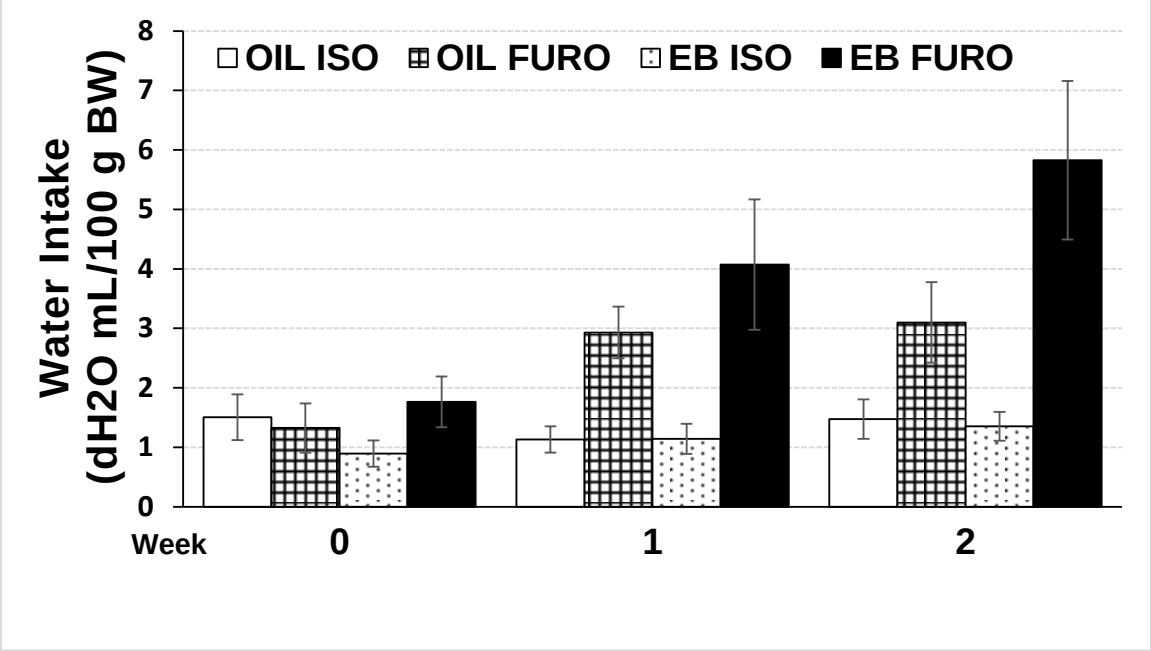


Figure 38. **2 h Water Intake:** Water intake/100 g body weight (BW) during a two hour test by OIL- (white bars; n = 7) and EB- (black bars; n = 7) treated rats. Testing began on day 4, 18-24 hour after the second injection with 0.15 M NaCl (ISO) or furosemide (FURO) using the multiple 18-24 h protocol. * = Water intake on week 1 was significantly less than that on week 1-2.

*Salt Intake*

Three-way repeated measures ANOVA revealed a main effect of drug [F (1, 23) = 33.7, p<0.001, $\eta^2$= 0.59], a main effect of time [F (2, 46) = 19.0, p<0.001, $\eta_p^2$ = 0.45], and a time by drug interaction [F (2, 46) = 14.0, p<0.001, $\eta_p^2$ = 0.38] for salt intake normalized to bodyweight (Figure 38) by OIL or EB treated rats given multiple FURO or ISO treatments. Pairwise comparisons of the interaction revealed salt intake increased after each weekly test by OIL (ps<0.05, 0.001, 0.01) and EB (ps<0.01, 0.001, 0.05) treated rats given multiple FURO treatments.

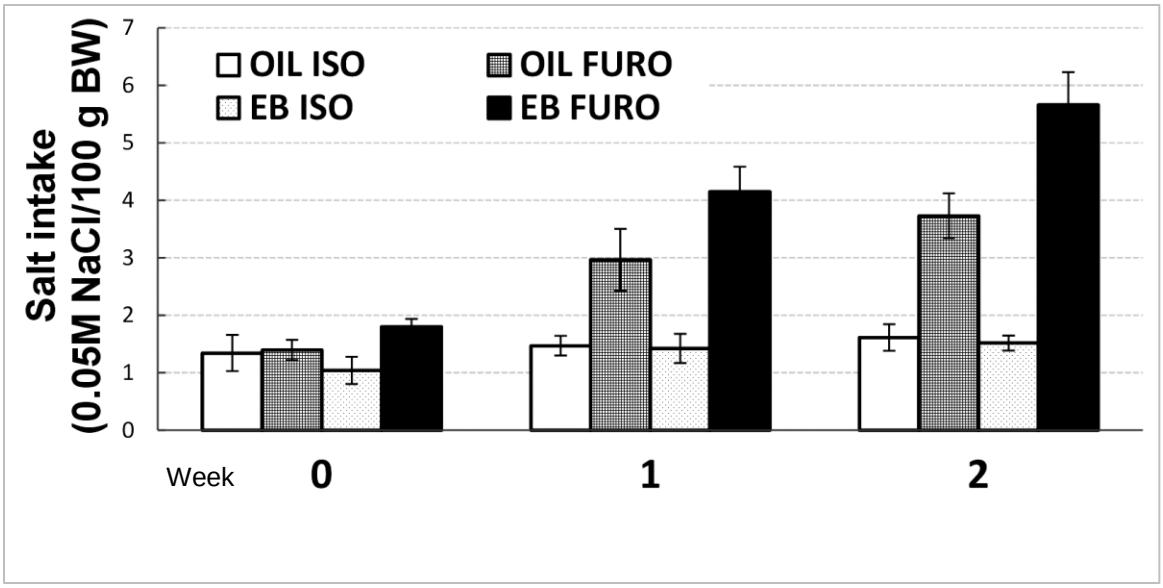


Figure 39. **2 h Salt Intake:** Salt intake/100 g body weight (BW) during a two hour test by OIL- (white bars; n = 7) and EB- (black bars; n = 7) treated rats. Testing began on day 4, 18-24 hour after the second injection with 0.15 M NaCl (ISO) or furosemide (FURO) using the multiple 18-24 h protocol. * = Salt intake on week 1 was significantly less than that on week 1-2.

*Glia Fibrillary Acidic Protein*

Three-way ANOVA revealed a main effect of drug [F (1, 48) = 4.3, p<0.05, $\eta_p^2$ = 0.08] on protein expression of GFAP after a single and multiple 18-24 h FURO, no main effects of hormone or frequency, and no interactions. Planned comparisons revealed that GFAP expression after a single 18-24 h FURO was significantly less (p<0.05) in EB-treated rats.

**Glia Fibrillary Acidic Protein after a Single 18-24 h and Multiple 18-24 h FURO**

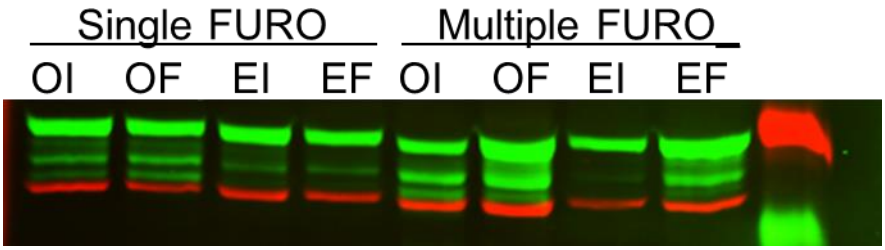


Figure 40. Representative immunoblot for GFAP (green) and beta actin (red) density in the DVC after a single 18-24 h FURO.

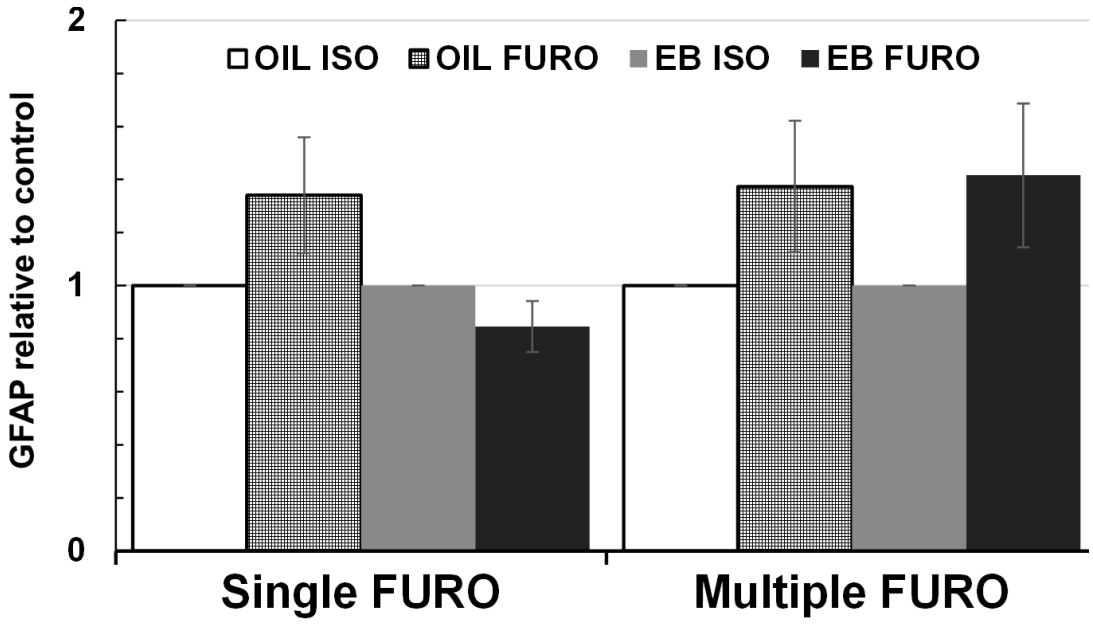


Figure 41. **Density of GFAP in the DVC.** GFAP density in the DVC of OVX rats given OIL (left) or EB (right) and a single treatment with 0.15 M NaCl (ISO; white bars) or furosemide (FURO; black bars) and then terminated 18-24 hours later.

*Calcium Calmodulin Kinase II*

Single 18-24 h FURO

Two-way ANOVA on protein expression of GFAP after a single 18-24 h FURO revealed no main effects of hormone or drug and no interactions. The change in expression of CaMKII from ISO was significantly decreased in EB-treated rats compared to Oil-treated rats ($t = 0.892.3$; $p<0.23$).

**Calcium Calmodulin Kinase II after a Single 18-24 h FURO**

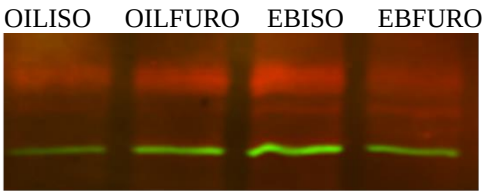


Figure 42. Representative western blot for CaMKII (red) and beta actin (green) density in the DVC

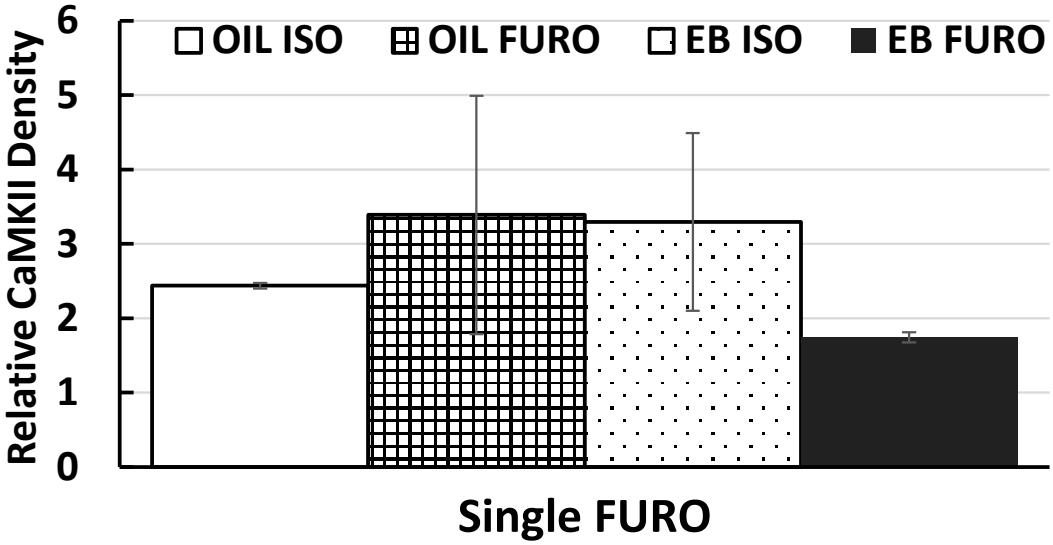


Figure 43. **Density of CaMKII in the DVC.** CaMKII density in the DVC of OVX rats given OIL (left) or EB (right) and a single treatment with 0.15 M NaCl (ISO; white bars) or furosemide (FURO; black bars) and terminated 18-24 hours later.

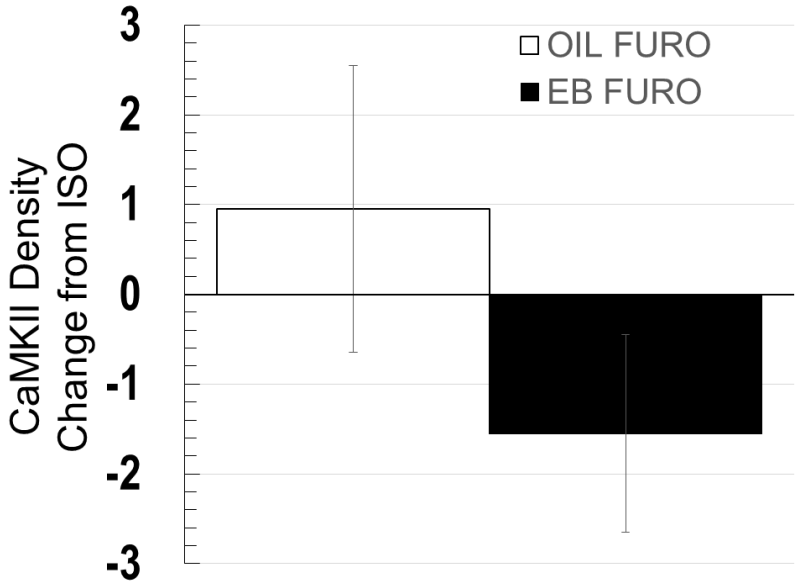


Figure 44. **Change in CaMKII density in the DVC.** CaMKII density in the DVC of OVX rats given OIL or EB and a single treatment with furosemide (FURO; OIL, black bar; EB, orange bar) expressed as change from CaMKII density after a single treatment with 0.15 M NaCl.

Multiple 18-24 h FURO

Western blot for the protein expression of CaMKII after multiple 18-24 h FURO resulted in weak to no signal detection, therefore it was not possible to reliably quantify. Unsuccessful band detection may be due to an inappropriate antibody concentration for the level of expression after multiple 18-24 h FURO. However, linear range analysis revealed the antibody for CaMKII was detectable in whole brain homogenate down to low concentrations of protein. Consideration must be given to differences between a whole brain homogenate containing brain areas where CaMKII expression may be more pronounced. CaMKII within the DVC may be expressed in low concentrations, making detection of changes in CaMKII after multiple 18-24 FURO treatments difficult. Western blot technique may not be the optimal technique for detecting proteins expressed in extremely low concentrations.

**Calcium Calmodulin Kinase II after Multiple 18-24 h FURO**

OILISO OILFURO EBISO EBFURO

Figure 45. Representative western blot for CaMKII (red) and beta actin (green) density in the DVC of rats after multiple 18-24 h FURO

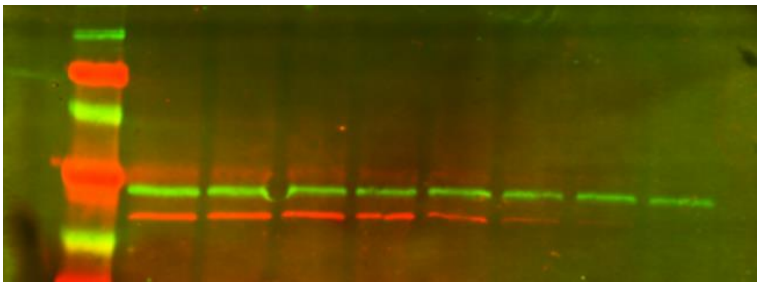

Figure 46. Representative western blot for the linear range of CaMKII (red) and beta actin (green) density in rat whole brain homogenate.

# CHAPTER V

# DISCUSSION

Repeated experiences with treatments that produce sodium loss increase sodium intake with each subsequent experience, a form of behavioral plasticity that is more pronounced in females with EB. We hypothesized that such plasticity occurs within central areas associated with the initial detection of sodium loss prior to integration by hypothalamic control centers. We tested this idea by first using immunohistochemistry to examine the time course of changes in astrocytes in the hindbrain dorsal vagal complex.

## GFAP-ir in the Area Postrema

In general, astrocyte abundance, as indicated by GFAP-ir, was decreased in the AP 18-24 hours after FURO treatment. Astrocyte abundance was also decreased in the rostral AP 2 hours after FURO independent of hormone treatment. The decrease in astrocytes in the caudal, middle, and rostral AP 18-24 hours after FURO (Figs. 9, 11, 13), at the time of salt intake, imply that these astrocytes may be sensitive to an osmotic signal associated with hypovolemia and/or hyponatremia. Astrocytes in the SFO, another sensory circumventricular organ, show expression of a sodium sensitive sodium channel capable of detecting increases in extracellular sodium

(Watanabe 2006) and astrocytes in other areas facilitate body water homeostasis by the expression of water channels or aquaporins (Roberta 2010). The functional implications of sodium channels and aquaporins has yet to be fully understood, but some believe astrocytes respond to osmotic shifts by swelling, a response thought to spare neurons from the damaging effects of hypertonicity (Schliess 1996). At present, it is unknown if astrocytes in the AP express any of these regulatory proteins. Thus whether the sensing capabilities of astrocytes in the AP have influence on neural excitability or inhibition is an area of active investigation with many questions remaining.

The effects of EB on astrocytes depended on level in the AP and were detectable 2 hours after FURO. Specifically, astrocyte abundance was decreased in the caudal and middle AP 2 hours after FURO in rats given EB compared to OIL-treated rats, but this difference was not observed in the rostral AP, where astrocyte abundance was decreased in both OIL- and EB-treated rats. The physiological response to FURO (e.g. increased urinary excretion) is a relatively rapid disruption of fluid balance, which activates compensatory cardiovascular and renal mechanisms. The loss of astrocytes in the caudal and middle AP at this time point suggests the effects of estradiol on astrocytes may promote early detection of such disturbances. The lack of an estradiol effect in the rostral AP may simply be due to differences in the astrocyte phenotype and/or function at this level of the AP. Alternatively, differential effects according to level within the AP may be due to the type of input, whether it is gastrointestinal in the rostral AP or cardiovascular in the caudal AP. It is also well known that steroid hormones initiate glial reorganization in areas of the brain involved in reproduction (Garcia-Segura 2006) and in astrocyte cultures EB inhibits GFAP expression (Rozovsky 2002). Thus, retraction of astrocytes in EB-treated rats in the AP may be another way in which hormones confer protection and alter the activity of neurons that project to other central areas, thereby initiating a more rapid behavioral response to sodium loss by EB-treated rats.

Astrocytes in the AP display unique morphology and functional characteristics compared to other astrocytes in that they do not contain some of the adhesion proteins involved in membrane formation and association with vascular endothelial cells (Willis 2007). It has yet to be determined the role these astrocytes play in neural humoral communication. One theory put forward by Willis *et al.* 2013 proposes that these astrocytes serve as an early response to maintain BBB integrity by essentially sensing the 'threat', sending out a distress signal, and then withdrawing from the site to allow infiltration of macrophages and repair cells. Support for this theory can be found in the fact that astrocytes in the AP have a wide variety of receptors for neurotransmitters and peptides (Carpenter 1988) and the discovery of immune cells (Goehler 2006) in the AP. Thus, astrocytes may participate in an immuno-chemosensory role to monitor changes in a wide variety of pathways. A specialized astrocyte population residing at the interface of central and peripheral communication would make sense given the need for the CNS to monitor the periphery and rapidly respond to protect the brain from peripheral threats.

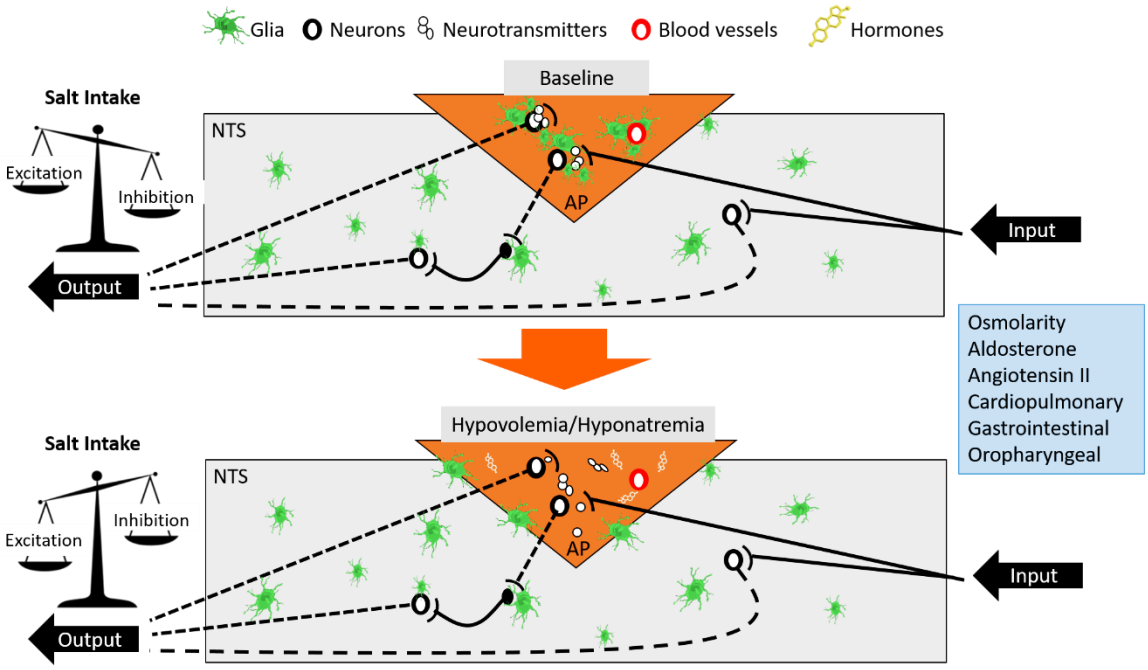


Figure 47. **Glial modulation of neuronal signaling in the DVC to influence salt intake.** Top figure illustrates glial association with blood vessels and neurons during baseline conditions when salt intake is inhibited. Bottom figure represents glia following changes in the input in response to hypovolemia/hyponatremia that may permit the excitation of salt intake.

**GFAP-ir in the Nucleus of the Solitary Tract**

Astrocyte abundance in the caudal and middle NTS did not change 2 hours after either ISO or FURO treatment, whereas GFAP-ir was decreased 18-24 hours after FURO treatment in both the caudal and middle NTS independent of hormone treatment. The NTS contains numerous sensory neurons and bundles of fibers that require glial support to connect to other areas in the brain. Therefore, the lack of any changes in astrocytes two hours after FURO when the incoming signals of hypovolemia are peaking is to be expected. The decrease in astrocytes 18-24 hours after FURO may indicate a change in signal transduction requiring less support or a local disruption in extracellular homeostasis.

**GFAP and Synaptic Protein Expression in the Dorsal Vagal Complex**

In contrast to the decrease in GFAP-ir found after a single FURO, the protein expression of GFAP in the DVC after a single or multiple 18-24 FURO was not appreciably different between ISO and FURO treated rats. This may due to using tissue punches encompassing a broad area, including the AP, NTS and nuclei in the surrounding areas, where visualizing differences are less likely given astrocytes are present in virtually all areas of the brain. Remarkably, the change in GFAP expression after a single 18-24 h FURO was greater in EB-treated rats compared to that in OIL-treated rats, suggesting a hormonal effect of estradiol on astrocyte abundance in the DVC. Looking at the change in GFAP expression after multiple 18-24 h FURO treatments showed no difference between the OIL- and EB- treated rats.

**Physiological Responses**

In order to measure physiological and behavioral responses to a body fluid challenge repeated multiple times without compounding to volume loss, blood samples were taken from rats used for immunohistochemistry and western blot rather than rats used in physiological and behavioral studies. Blood was collected at termination for plasma analysis of volume and electrolyte status, as repetitive blood sampling may disrupt body fluid balance and instigate a new source of stress in the animals. Nonetheless, terminal measures of the plasma revealed increased plasma protein and hematocrit after FURO treatment, indicating the loss of fluid volume., Hypovolemia was indirectly inferred from the urine volume loss and decreased body weight that accompanies FURO-induced hypovolemia as reported previously by us and others (Curtis 2017; Rabe 1975). In other words, furosemide was an effective diuretic as demonstrated by the volume of urine loss (Fig. 21) and the corresponding body weight loss (Fig. 20) 1- 2 hours after furosemide. Furosemide treatment maintained its effectiveness with each treatment, producing comparable urine volume losses between subsequent weekly treatments. EB treatment had no effect on the diuresis even though EB-treated rats had less urine output than OIL-treated rats after the first FURO treatment. EB is known to decrease body weight (Fig. 18) and EB-treated rats weighed less than OIL-treated rats (BW before FURO: EB = 296; OIL = 308); therefore it is possible that EB-treated rats had less volume to lose.

Furosemide was also an effective natriuretic, causing sodium excretion (Fig. 22) 1-2 hours after furosemide, as determined by measuring the concentration of sodium in the excreted urine. Sodium excretion was greatest on week three, most likely due to random variability as doses of furosemide were calculated based on body weight and did not differ. Furosemide also led to the excretion of potassium (Fig. 23) 1-2 hours after furosemide that was greatest on week

three. Urine osmolality (Fig. 24) was not different after FURO. Furosemide blocks reabsorption of sodium at the loop of Henley leading to increased sodium in the distal tubule. An increase in sodium concentration in the distal tubule reduces water reabsorption, thus both water and sodium are excreted. The increase in sodium in the distal tubule of the kidney also increases sodium reabsorption in this segment in exchange for potassium, thus potassium is also excreted with sodium and can potentially lead to hypokalemia if the natriuresis continues. In general, filtration of sodium and potassium were comparable between FURO treatments indicating kidney function remain unchanged with each FURO treatment and EB had no effect on renal handling after FURO treatment.

In order to facilitate hyponatremia, rats were food deprived overnight and only allowed access to water. Overnight urinary excretion after FURO did not differ from ISO treated rats and was not different between FURO treatment weeks. Overnight excretion of sodium and potassium and urine osmolality in FURO treated rats also did not differ from ISO treated rats and was not different between FURO treatment weeks. Together, these findings demonstrate that renal handling of sodium and potassium was restored to pre-FURO levels during this time. Surprisingly, plasma sodium was unchanged after FURO despite the increase in sodium excretion, however, the decrease in plasma volume facilitated the maintenance of plasma sodium concentrations. Plasma potassium was decreased, mirroring the increase in potassium excretion. Plasma osmolarity increased after FURO due to the concentrating effect of hypovolemia and the unchanged plasma sodium concentration. Apparent hyponatremia may require additional water intake over time to increase the dilution of the extracellular fluid, as demonstrated by Falk in 1965. Still, rats increased their overnight water intake after FURO compared to the week of ISO treatment, validating previous work showing hypovolemia stimulates water intake (Gilman 1937, Stricker 1966, Zimmerman 1981).

As an additional determination of the effectiveness of FURO to produce hypovolemia/hyponatremia, the fluid status of the rats was determined by calculating sodium and water balance after each FURO treatment. FURO treatment resulted in negative sodium balance after each treatment, and consistent with urine sodium excretion on week three, there was a significantly greater decrement in sodium balance on week three than that on week two and four. Rats treated with FURO also were in negative water balance after each treatment and it did not differ between treatments. Therefore, FURO produced a significant body fluid challenge by depleting water and sodium.

**Behavioral Responses**

In response to water and sodium depletion, rats treated with FURO and then given a 2 hour 2-bottle intake test increased their water and salt intake compared to rats treated with ISO (Figs. 38 and 39). Interestingly, EB-treated rats did not have greater salt intakes than did OIL-treated rats. The absence of a hormonal effect of salt intake was unexpected. However previous studies used intact female rats without accounting for hormone treatment (Sakai 1989). Methodological differences may factor into the observed differences as well. The use of different methods of sodium depletion (e.g. sodium deficient diet vs. diuretics) and temporal differences in protocols result in contradictory findings. Rats permanently housed in metabolic cages with testing conducted in home cages compared to rats transferred into metabolic cages for testing may behave differently even with an acclimation period (Tarland 2007).

Overall, it may be said that furosemide-induced sodium depletion was an effective stimulus for producing the physiological responses to water and sodium loss. Rats with and without hormone treatment responded to furosemide equally, resulting in negative water and sodium balance. Similarly, given there were no differences in excretion of volume or sodium

demonstrates the renal compensatory mechanisms to water and salt loss were comparable between the groups. Therefore, differences in the central processing of signals related to sodium depletion are not due to differences in renal handling.

The decrease in the expression of GFAP and the parallel decrease in CaMKII may signify a relationship between the two proteins. Glia that do not express GFAP may effectively be 'silent,' inactive, or possibly absent, thereby incapable of performing their role in maintaining the synapse and neurotransmission. For instance, if they are unable to provide neurons with calcium, the proteins involved in calcium dependent exocytosis of neurotransmitter may be inhibited. In effect, excitatory or inhibitory neurotransmission may be blunted. Considering NTS neurons are known to provide an inhibitory drive on the central circuit stimulating salt intake, it may be that without active glia in the AP and NTS to support neurotransmission, that inhibition is removed, thus salt intake ensues. Future studies will consider this avenue with the goal towards characterizing the relationship between glia and neurotransmission.

In essence, these findings are consistent with our hypotheses and suggest that the behavioral plasticity observed in salt intake may be, in part, regulated by brain plasticity stimulated by hypovolemia/hyponatremia and involving estradiol influences on astrocytes in the dorsal vagal complex. Accordingly, one might predict that preventing the retraction of astrocytes from the DVC and thereby preventing passage of any signal of the disturbance into the CNS may prevent the stimulation of salt intake. This idea has yet to be tested. However, the indication that glial remodeling has functional significance was shown in the supraoptic nucleus of the hypothalamus where the release of oxytocin was directly related to a reduction in astrocytic coverage of neurons (Chapman 1986; Theodosis 1986). It was later determined that the retraction of astrocytes allowed for free diffusion of neurotransmitters to access neurons (Nicholson & Sykova 1998). Remarkably, it has also been shown that glial remodeling occurs throughout the reproductive cycle (Garcia-Segura *et al.* 1994) and that estrogen itself can induce changes in

astrocytes of the SON (Theodosis and Langle 2006; Theodosis *et al.* 2006). Identifying estrogen receptors on astrocytes in the DVC will further studies where blocking ER's may demonstrated an attenuation in glial remodeling and the detections of peripheral signals due to sodium depletion. Again, astrocytes in the AP seem to express a differing morphological and functional profile than other astrocytes and thus could play a more active role in neural communication. While cells can be positive for GFAP, which identifies them as astrocytes, this doesn't distinguish one astrocyte from another nor does it identify all astrocytes. Investigations into the unique properties of astrocytes in the AP will require the development of antibodies to distinguish specific astrocyte populations.

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